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Viral synthesis in Escherichia coli B infected with ultraviolet irradiated and unirradiated bacteriophage

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VIRAL SYNTHESIS IN ESCHERICHIA COLI B. INFECTED WITH ULTRAVIOLET IRRADIATED AND UNIRRADIATED BACTERIOPHAGE

by

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INTRODUCTION

Interest in the Study

When a bacteriophage of the T series infects its host bacterium, Escherichia coli strain B., under suitable conditions, the bacterium will lyse after a certain period of time, and a number of new bacteriophage particles will appear which are genetically identical to the infecting bacteriophage. Replication of the bacteriophage has taken place. To understand the mechanism of this replication process is one of the chief aims of bacteriophage research.

One significant step in the development of new phage was that discovered by Hershey and Chase (1). They showed, in working with T_2, that the deoxyribonucleic acid (DNA), which makes up about 50% (2) of the infecting phage particle, is injected into the bacterium upon adsorption of the phage, and that the protein coat remains outside and attached to the bacterial cell. They found that most of this could be mechanically sheared off without affecting the phage replication process. This indcates that the genetic markers of the phage particles are carried by the DNA unless some small component has escaped notice.

The maximum absorption of DNA lies near 2600 $\mathring{A}$, close to the 2537 $\mathring{A}$ mercury line which is commonly used as a source of ultraviolet radiation. This radiation produces chemical changes in the DNA (3, 4) and the action spectrum for the inactivation of T_2 resembles in this region the

absorption spectrum of DNA (5). Luria (6) found that UV irradiated T_2, which have a very low survival as far as single infection is concerned, are able to initiate phage synthesis upon multiple infection. These facts indicate the possible usefulness of employing ultraviolet (UV) irradiated bacteriophage to infect bacteria in order to study bacteriophage synthesis, and also point to the important role DNA may have in this study.

This thesis describes studies on bacteriophage synthesis upon multiple infection of bacteria with UV irradiated bacteriophage, with infection with unirradiated bacteriophage as control. The synthesis of DNA, together with the appearance of active intracellular phage, are used to follow the process of development.

Brief Review of Literature on the Use of UV Irradiation with Bacteriophage

The most obvious result of irradiating phage particles with ultraviolet light is that a large percentage of the particles become inactive in the sense that they do not give rise to plaques, when plated on agar plates with sensitive bacteria and incubated, as do active phage particles. The inactivating effect may be indicated by means of survival curves which are obtained by plotting the logarithm of the fraction of particles active after a dose D of radiation, which is a function of time, with other parameters constant. In the cases of the T even phages, the survival curve is very nearly a straight line indicating that the inactivation is the result of a single "hit" (7).

Ultraviolet irradiation was used by Luria and Latarjet (8) to study the process of phage multiplication during the latent period. They irradiated infected bacteria at various times after infection and plotted survival curves for infective centers. Instead of the typical multiple-target type curves which they expected, they got (using T_2) continual decreases in the ultra-violet sensitivity until a little less than half way through the latent period, after which the UV sensitivity increased and the curve developed a multiple target shape. Later, Benzer (9) who improved the technique, did similar experiments with similar but more consistent results with T_2. He also did experiments of this type with T_7 bacteriophage and found survival curves which showed an increase in target number during the first half of the latent period as well, which is the result Luria and Latarjet had anticipated for T_2. This indicates differences in the T_2 replication process compared with that of T_7.

It has been found for several of the phage particles (10) that the latent period is somewhat longer in the case of survivors of UV irradiation. In many instances, however, UV irradiated phage particles perform the same function and exhibit the same phenomena as unirradiated phage. Some of the more noteworthy examples are as follows: adsorption to the host cell, killing of the host as a colony former, exclusion of the genetic material from superinfecting phage types (11), stimulation of the cell to breakdown of the superinfecting particles (12), production of lysis inhibition in cells previously infected with r+ types (13), and lysis from without (14). That each of these phenomena do exist for irradiated as well as for unirradiated bacteriophage has been verified in connection with the experiments which will be described in this thesis.

Review of Literature on Multiplicity Reactivation

As has already been mentioned Luria (6) discovered that the survival of cells multiply infected with UV irradiated T_2 is much greater than one would expect if the phage particles infecting a cell are independent of one another. In the latter case the probability of survival depends on the survival of at least one of the infecting particles. One would expect a survival curve of a multiple-target type modified by the variation in numbers of phage infecting each cell. This extraordinarily high survival was found for multiple infection with phage T_4, T_5, and T_6 as well as for T_2. The suggested explanation for this effect was that each phage particle contains a number of ultraviolet sensitive genetic units, damage to any of which is lethal for single infection. The undamaged units from the separate infecting particles in some unknown way multiply and combine to form new phage particles. This has been called the "recombination theory of multiplicity reactivation."

Luria and Dulbecco (15) later extended these experiments with T_2, T_4, and T_6 and were able to get fairly close agreement between experimental survival curves and curves computed for a specific number of genetic units per phage particle for survivals down to 10-3. The agreement was good enough to lend support to the correctness of the basic ideas of the recombination theory.

Dulbecco (16) carried out multiplicity reactivation experiments with T_2 using improved techniques enabling him to extend the survival values down to 10^{-7}. Upon so doing, he found that the slope of the experimental

survival curves in this extended region was not what was predicted by the recombination theory. The recombination theory as an explanation for multiplicity reactivation would have to be discarded or at least be subjected to a major revision.

The deviations from the Poisson distribution of phage among the bacteria due to the presence of long filamentous bacterial cells was suggested as the reason for the failure of the recombination theory to explain multiplicity reactivation satisfactorily (17). More recently, however, (18) this was checked experimentally by performing multiplicity reactivation experiments upon cells remaining after the long filamentous cells had been removed. The results of these experiments were similar to those of Dulbecco and indicated that the presence of the long filamentous cells had not been responsible for the failure of the recombination theory. Instead, another hypothesis has been suggested; if it is assumed that about 60% of the damage due to UV irradiation is completely reactivable, but 40% is much less reactivable--not at all in the straight line portion of the curve, the recombination theory satisfies the requirements for explaining multiplicity reactivation for survivals down to 10^{-7}. It has been found recently (19) that under proper conditions reactivation also takes place in T_2 after inactivation with ionizing radiations with an efficiency comparable to that found after UV inactivation. Since the action of ionizing radiations is quite different from that of UV, such a discovery lends strong support to a replacement hypothesis.

Review of Literature on Photoreactivation

The phenomenon of photoreactivation was first discovered in connection with Streptomyces griseus conidia by Kelner (20) but was discovered accidentally a little later in bacteriophage by Dulbecco (21). Part of the inactivation caused by the ultraviolet light is reversed by exposure to longer wave length radiation after the phage have become adsorbed to sensitive host bacteria. After a thorough description of photoreactivation by Dulbecco (22), studies of the kinetics of photoreactivation were made by Bowen (23, 24) with T_2r.

Brief Review of Literature on the Synthesis of Phage DNA

After infection of a bacterium by a bacteriophage, protein synthesis continues, the synthesis of RNA is stopped, and the synthesis of DNA for new bacteriophage is begun (25). Even the adsorption of UV irradiated T_2r+ is found to inhibit RNA synthesis (26). The synthesis of the new DNA may be followed to a certain extent by an assay for the total DNA. One of the pyrimidine bases, cytosine, which had been found present in all DNA's, was found to be absent in bacteriophage T_2 (27) and later in the other T even phages, although it was present in host cell DNA. Wyatt and Cohen (28) found a new base, hydroxymethyl cytosine, to be present in the DNA's of these phages. Hershey and his coworkers later used this fact in a study of T_2 phage precursor nucleic acid (2).

Many T_2 infected cultures were used. Each was stopped at a particular time after phage infection and the amounts of phage DNA and bacterial DNA were estimated by separating the bases cytosine and hydroxymethyl cytosine on 2 dimensional paper chromatograms following isolation and acid hydrolysis of the DNA. Since each point on the curves for bacterial DNA and phage DNA versus time was from a separate culture, the points for a given time after infection varied widely, but averages of the points showed a gradual decrease in bacterial DNA over a period of 30 minutes and an increase in T_2 DNA after a short lag period.

Another new base was discovered by Sinsheimer (29) in the DNA from T_2r+. By means of ion exchange chromatography, a double peak was found where the component hydroxymethyl cytidylic acid was expected to produce a single one. Upon further investigation it was found that one of the peaks was due to hydroxymethyl cytidylic acid while the other one was due to a glucose substituted hydroxymethyl cytidylic acid. The glucose molecule is probably attached in the hydroxymethyl part of the base.

Review of Literature on the Use of Chloramphenicol and 5-Methyl Tryptophane with Phage

Several years ago 5-methyl tryptophane was found to exhibit bacteriostatic effects which could be blocked by tryptophane (30). It was found to inhibit T_2 or T_4 multiplication in bacteria without affecting oxygen consumption, but this inhibitory effect was reversed on addition of tryptophane (31). Fildes and Rydon (32) found that 5-methyl tryptophane is

inhibitory because it competes with tryptophane at sites of protein synthesis.

More recently, a concentration of 60 μg per ml. of chloramphenicol, an antibiotic, was found (33) to suppress growth and protein synthesis without inhibiting either RNA or DNA synthesis in Escherichia coli (E. coli). Since one can postulate that induced biosynthesis of enzymes necessary for hydroxymethyl cytosine synthesis follows virus infection, Rosenbaum et. al. (34) attempted to determine whether chloramphenicol blocks the development of phage DNA in infected E. coli cells thus indicating a need for protein synthesis. They concluded that the virus carries the necessary enzymes for HMC synthesis or that they are already present in the cell, since they found some phage DNA synthesis after adding chloramphenicol before or with the infecting phage. Tomizawa and Sunakawa (35) found no DNA synthesis if chloramphenicol was added from 0 - 2 minutes and concluded that the reason for the results of Rosenbaum et al., was that the low concentration of the antibiotic used by these authors was inadequate to inhibit protein synthesis.

Conversely, when Tomizawa and Sunakawa added chloramphenicol after phage DNA synthesis had begun, this DNA synthesis was not stopped but continued at a constant rate--the rate of synthesis at the time the chloramphenicol was added. Burton (36) working with 5-methyl tryptophane found similar results; the addition of 5-methyl tryptophane at phage infection prevented any phage DNA synthesis, whereas the addition of 5-methyl tryptophane at some later time after the DNA synthesis had begun, served only to keep the rate of synthesis then present from increasing.

MATERIALS, APPARATUS, AND METHODS

Preparation of Phage Stocks

In performing experiments in which bacteria are infected with phage at phage to bacteria ratios in the range of 3 to 8, especially in one or two liter amounts of bacteria at concentrations of approximately 10^9 bacteria per milliliter, sizeable quantities of phage at fairly high concentrations are required. In order to have the phage available it is best to grow a few hundred milliliters of phage at one time in as high a concentration as possible.

Since it had been shown in previous experiments in our laboratory (37) that high titers of phage could be achieved from phage infected bacteria grown in Fraser medium, this medium was almost always used in producing phage for stocks in this set of experiments. The formula for Fraser medium will be given along with others in the next section.

The Fraser medium is autoclaved in the flask which is to be used for growing the bacteria and phage. The E. coli B, which is the host cell for the phage used in these experiments, is aseptically added to the medium at a final concentration of approximately 10^7 bacteria per milliliter. Aeration is provided while the flasks are kept at a temperature of 37° C.

When the concentration of the E. coli B reaches approximately 2×10^9 bacteria per milliliter, the phage are added to a final concentration of

2×10^8 phage per milliliter. In this way only about one tenth of the bacteria are initially infected. At the end of the first latent period when the first burst takes place enough phage are released so that each of the remaining bacteria is multiply infected (or infected with two or more phage particles). Since the phage used are of the r+ type, lysis inhibition is exhibited and the complete lysis of the cells is not observed until six to ten hours after infection. If Dow-Corning antifoam A is added to control foaming, complete lysis may be delayed until twelve to sixteen hours. Aeration is stopped and the flasks removed from the constant temperature baths when lysis is essentially complete.

The lysate is stored in the cold room briefly until the phage can be cleaned up and concentrated for use as stocks. Differential centrifugation is used for cleaning the phage. The first centrifigation of the raw lysate is a low speed run in order to spin out bacterial debris. The angle centrifuge run at speeds of four to six thousand revolutions per minute is sufficient for this procedure. After centrifugation the supernatant is poured off and kept for a high speed centrifugation. The sediment is resuspended in phage buffer (described in the next section) and spun at low speed again. The supernatant is poured off and combined with the supernatant of the previous run while the sediment is discarded.

During centrifugation an effort is made to keep the centrifuge tubes as sterile as possible. The purpose of the next centrifugation procedure is to spin down the phage particles themselves discarding anything smaller with the supernatant. Ten or twelve thousand revolutions per minute is satisfactory for this operation. The supernatant is poured off and

discarded. Two or three mls. of phage buffer are added slowly to the slanting inside edge of each centrifuge tube, the tube is swirled lightly rinsing the pellet and the liquid is poured off and put with other batches of phage which have not been centrifuged at 10,000 - 12,000 r.p.m. To each tube, which contains a pellet of sedimented phage, six mls. of phage buffer are added. The tubes are left to stand in the cold room for two or three hours which tends to resuspend the phage to a certain degree, and then a stirring rod is used to disperse the phage into a homogenous suspension. The contents of the tubes are pooled in a sterile flask, mixed thoroughly and then centrifuged once more at the lower speed. The supernatants are pooled in a sterile flask and constitute a homogeneous phage stock of usually 4 - 9 x 10^{11} phage per ml.

After a phage stock is used for several days the turbidity of the suspension may begin to noticeably increase in spite of the fact that it is stored in the cold room. For this reason stocks of phage are grown only in large enough quantities to provide sufficient innocula for a two to four week period. They are replaced before this time should it become necessary.

In a method used earlier in cleaning phage for stocks, the phage are given a second centrifugation at 10,000 - 12,000 r.p.m. These are resuspended in buffer and used for stocks, but it is very difficult to get a good homogeneous suspension of phage the second time. In the process of resuspending the phage many are broken and the released DNA makes the suspension very viscous and non-homogeneous.

Preparation of Media

The media used in these experiments are, with few exceptions, media commonly used in the culture of bacteria. The liquid media used for bottle cultures are tryptone broth, Fraser medium, and a salts-glucose medium known as M-9 medium. In a few cases there are modifications of the basic formulas which will be indicated in the instances these are used. In addition to these is the buffer which is used in serial dilution techniques as well as in resuspending phage for phage stocks. The formulas used in making up these media are listed below.

Tryptone broth

10.0 gms.	Bacto-tryptone
5.0 gms.	Na Cl
1.0 liter	Distilled water

Fraser medium

4.5 gms.	KH_2PO_4 (anhydrous)
10.5 gms.	$NA_2H\ PO_4$ (anhydrous)
3.0 gms.	NH_4Cl
0.3 gms.	$MgSO_4 \cdot 7H_2O$
30.0 gms.	Glycerine (24 mls.)
0.3 mls.	1 M. $CaCl_2$
15.0 gms.	Casamino acids (Difco)
3.0 mls.	1% gelatin
1.0 liter	Distilled water

M - 9 medium

3.0 gms.	KH_2PO_4 (anhydrous)
6.0 gms.	Na_2HPO_4 (anhydrous)
0.5 gms.	NaCl
1.0 gms.	NH_4Cl
0.2 gms.	$MgSO_4 \cdot 7 H_2O$
4.0 gms.	Sterile 10% glucose (added after autoclaving)
1.0 ml.	1% gelatin
1.0 liter	Distilled water

Phage buffer

3.0 gms.	KH_2PO_4 (anhydrous)
7.0 gms.	Na_2HPO_4 (anhydrous)
4.0 gms.	NaCl
0.2 gms.	$MgSO_4$
10.0 mls.	1% gelatin
1.0 liter	Distilled water

The agar solution poured as a bottom layer into Petri dishes for assay purposes with both bacteria and phage, and the softer top layer used also in this connection, are made up in preparation for many of the experiments. The bottom agar used contains tryptone and glucose and is known as T-G bottom agar.

T-G bottom agar

11.5 gms.	Bacto-agar
10.0 gms.	Bacto-tryptone
8.0 gms.	NaCl

1.5 mls.	1 N . NaOH
10.0 mls.	Sterile 10% glucose (added after autoclaving)
1.0 liter	Distilled water

Top agar

1.5 gms.	Bacto-tryptone
1.05 gms.	Bacto-agar
0.75 gms.	NaCl
150.0 mls.	Distilled water

In experiments where it is desired to know the average number of whole phage per bacterial cell at a particular time, even previous to the lysis of the bacteria, it is useful to artificially lyse the cells by placing an aliquot of the suspension in a lysing medium. The formula for this medium follows.

Lysing solution

.195 gms.	KCN
10.0 mls.	Chloroform
290.0 mls.	Phage buffer

Further details on the use of this medium will be given when experiments which make use of it are described in a later section.

Culture Tubes, Flasks and Bottles

The bacteria and phage used in these experiments are grown in pyrex glass tubes, flasks, or bottles, with the volume of material needed determining which of the three types of containers are employed. The tubes

used are large, rimless test tubes of approximately 50 mls. capacity, whereas the flasks used are 300 or 500 ml. Erlenmeyer flasks. Both two-liter and four-liter bottles are used in various large-scale experiments. In the test tubes a piece of glass tubing drawn down to a small opening at the bottom end is inserted through the cotton plug at the top. This provides a means of bubbling air at low pressure through the culture. In the culture bottles rubber stoppers are used. Air is supplied through a tube with a coarse sintered glass cylinder at the bottom. A straight tube with a cotton plug also is inserted through the stopper as well as an L-shaped piece of tubing. The former is used when adding or removing samples while the latter acts as an exhaust tube for the outgoing air. The culture flasks are provided with cotton plugs with tubes extending through. In the smaller flasks the same provision made for aeration as is made for the test tubes is sufficient, whereas in the larger flasks the three tubes as described in connection with the bottles are used.

An overnight culture of E. coli B., grown in an aeration tube in a 37° C. constant temperature bath, serves as an inoculum for the culture tubes, bottles, and flasks used in each of the experiments. Colonies of E. coli B. on slants stored in the refrigerator serve as inocula for the overnight cultures.

Ultraviolet Irradiation

Irradiation of the phage is provided by two 8-watt G.E. germicidal lamps mounted 50 cm. above the top of the sample being irradiated. The phage are exposed to the ultraviolet light in 10 ml. amounts in the bottom of a Petri dish at concentrations of 5×10^{11} phage per ml. or less. T_2 r+ phage exposed to the radiation in this manner have a survival of approximately 0.003 when the time of exposure is in the neighborhood of 35 seconds.

Sample Extraction Apparatus

Since most of the experiments in this group involve measurements as a function of time after infection of the bacteria by the phage, it is necessary to have apparatus available to withdraw samples from the cultures after specific intervals of time. The requirements are that the samples must be withdrawn quickly and that the volume must be measured quite accurately.

0.1, 1.0, and 5.0 ml. pipettes serve quite adequately in instances where no larger quantities than these are required.

In two of the experiments, samples 120 mls. in volume are extracted each time. In this case a long test tube with a side-arm attached and having a diameter of 5.0 cm. is used to collect the samples. A scratch, drawn on the tube at the meniscus of a known volume of 120 mls., serves to indicate the desired volume. The side-arm is attached to a water

aspirator with a trap in the line between. A glass Y is also inserted in this line with two of the three connections in the line as indicated. When the desired volume has been reached, a pinch clamp on a small piece of rubber tubing fastened to the third arm of the Y is opened. This serves to shut off the flow of sample by releasing the vacuum. A glass tube inserted through a rubber stopper in the top of the collecting tube is connected through a flexible tube to a long piece of glass tubing which is inserted into the desired culture bottle. The adjustment of the flow of water through the aspirator acts to control the rate of extraction of the sample.

With this apparatus samples may be extracted within 20 to 30 seconds. The samples are poured quickly into 0.1 volume of 3.0 M. trichloroacetic acid (T.C.A.) which stops all biological activity, and the time of the contact of the sample with the T.C.A. is indicated as the time for the particular sample involved.

Drying Apparatus

In two steps in the procedure for preparing samples for paper electrophoresis it is necessary to evaporate or concentrate the material to dryness. The drying apparatus used consists of an air heater, designed to raise the temperature of the air to 65° C. and two glass manifolds containing 10 glass jets apiece, which are the proper distance apart to fit into ten test tubes in two rows in a standard wire test tube rack. The

heater is connected to the manifolds through a Y.

The heater is made by winding a helical coil of "advance" wire of the proper gauge and length around a piece of glass tubing. Asbestos paper is used to cover the outside of the heater. A previously calculated A.C. potential is provided across the coil for a particular air flow rate.

In operation, the tubes with the samples being dried are placed in a 65° C. constant temperature bath. Air is supplied to the heater by a high pressure air line.

Estimation of Total DNA

In estimating the total amount of DNA in a sample a modification of the Dische diphenylamine test (26) is used. The reagent consists of 1 ml. of diphenylamine reagent dissolved in 98 mls. of glacial acetic acid with 2 mls. of concentrated H_2SO_4 added.

To the cold 5% T.C.A. precipitate in a small test tube is added 1 ml. of distilled water. 2.5 mls. of the reagent solution are added and the tubes are heated in a boiling water bath for 9 minutes. After the solutions are cooled, each is added to a spectrophotometer cuvette and the optical density at 600 mμ (D_{600}) is read against water. One ml. samples of blanks and standards are heated along with the unknowns after the addition of 2.5 mls. of reagent solution to each. The blanks are simply 1 ml. of distilled water; the standards are prepared by adding 200 λ's of a thymus DNA solution of 0.625 mg. per ml. to 0.8 ml. of distilled water. The average D_{600} of the blanks is subtracted from each of the

sample and standard readings and the corrected D_{600} readings of the samples compared with those of the corrected standards. The thymus DNA is a secondary standard which has been compared in a Dische diphenylamine test with a phage DNA of known phosphorus content. The thymus DNA solution, stored in the cold room, is very stable over long periods of time.

Assay of Bacteria and Bacteriophage

It is not necessary to mention in detail the steps used in assaying bacteria and phage cultures since the standard procedures used in this field are used. The agar layer technique as outlined in Adams (38) is used for assaying both phage and bacteria.

Paper Electrophoresis

As stated in the Introduction, T even phage DNA differs from E. coli B. DNA in that there is no cytosine in the DNA of T_2, T_4, and T_6 phage. The DNA of these phage does contain hydroxymethyl cytosine, however. In the E. coli B. DNA cytosine appears as one of the bases but no hydroxymethyl cytosine is found. This makes it possible to assay for the phage DNA and the bacterial DNA separately in a system containing both of these nucleic acids by assaying for cytosine and hydroxymethyl cytosine.

In one method it is first necessary to isolate the DNA mixture, then to hydrolyze the DNA to its purine and pyrimidine bases, and finally to

separate the bases and assay for the amounts of cytosine and hydroxymethyl cytosine present (2). One method for separating these two bases preparatory to assaying them is by means of paper electrophoresis. This method lends itself especially well to small amounts of material.

If the bases cytosine and hydroxymethyl cytosine as well as thymine, adenine, and guanine* are placed in a buffered solution at a pH in the range of three to five, the degree of dissociation of each amino group on each of the bases may be different (except for T which has no amino group). When an electric field is applied to this solution the bases, except for T, will migrate with different velocities in the same direction. This migration will be toward the negative electrode since in this pH range the amino group will become positively charged by the addition of a proton.

According to simple electrophoretic theory a solute of effective charge Q will have a force acting on it equal to $Q \times \frac{\partial \psi}{\partial x}$ which will be opposed by a frictional force equal to f x dx/dt where f is the frictional coefficient and dx/dt is the velocity of migration i.e. $F = Q \frac{\partial \psi}{\partial x} = f \, dx/dt$. The electrophoretic mobility U is defined as the velocity per unit potential gradient, or

$$U = \frac{\frac{dx}{dt}}{\frac{\partial \psi}{\partial x}} = \frac{Q}{f} \quad .$$

The frictional coefficient f is proportional to the average cross sectional area A which the solute particle presents as it migrates through the

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*Hereafter the bases cytosine and hydroxymethyl cytosine, thymine, adenine and guanine will be abbreviated as C, HMC, T, A and G respectively.

solvent. Since A is proportional to r^2 which is in turn proportional to $V^{2/3}$, where V is the volume, and since for constant density V is proportional to the mass M, f will be roughly proportional to $M^{2/3}$.

The problem was to find a pH at which the electrophoretic mobility is different for each of the bases mentioned and especially to separate the C and HMC from the other bases. In case the effective charge happens to be nearly the same for cytosine and for hydroxymethyl cyto- since the f value for the two bases would have to be different. Since the molecular weight of cytosine is 111, and that of hydroxymethyl cystosine is 141, the ratio of these each to the two-thirds power gives 0.85 for the ratio of f for C to f for HMC, and HMC should move 0.85 times as far as C in a given time.

Preliminary experiments to establish procedure

A solution of tris (hydroxymethyl) aminomethane-formic acid buffer* at pH 3.41 and 2.0 M. in formate was first made up to use as the buffer stock solution in a paper electrophoresis experiment with these bases, since at this pH the amino groups of the bases should all be ionized. Succeeding experiments proved this to be a satisfactory buffer.

After experimentation with dilutions of the concentrated buffer stock, 0.1 M. in formate of tris formate buffer was found to give little trailing of solute bands without excessive current.

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*Tris (hydroxymethyl) aminomethane formic acid buffer will be abbreviated to tris formate buffer.

Two preliminary experiments were done; one with C and HMC, the other with HMC and a C, T, A, G mixture. These will be described in some detail in the order listed.

Solutions of C (1.85 mg/ml) and HMC (2 mg/ml) were first made up and 20 λ samples of each were spotted, a small amount at a time, on a piece of electrophoresis paper* in two spots, each 10 cms. from one end. The spots were air dried between applications and in this way their size was kept to approximately 6 mm. in diameter. The paper was then wet gradually with buffer applied by means of a 5 ml. pipette, and then placed in the electrophoresis apparatus**. A potential was then applied between the two electrodes for a given period of time. This procedure was repeated for other potentials and other times. At the end of the selected time the potential was turned off and the papers taken off and air dried. In order to locate the new positions of the migrated bases, the papers were observed under a portable UV lamp which emitted primarily radiation of 2537 Å wave length, where the bases quench the fluorescence of the paper.

In these experiments with C and HMC, it was found that there was not so much trailing of the bands at 1000 V. as for lower voltages. At 1000 volts, the C band moved approximately 29 cm. from its original

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*The paper used was 2 1/2" x 18 1/4" Whatman 3 MM filter paper.

**A commercial model manufactured by the E-C apparatus company. (The apparatus itself consists essentially of two electrodes, one in the bottom of each of the two buffer compartments, an arrangement for bringing the liquid to the same level in the compartments, a cooling coil above and one below the paper which are connected to a cold water supply to conduct heat away, and a power supply with a variable output of from 0 to 1000 volts D.C.)

position in 2 1/4 hours. When correction was made for the endo-osmotic effect (an unionized base moved 6 cm. also toward the cathode), the ratio of the distance the HMC band moved to that which C moved was found to be 0.82. It was possible, then, to cut these bands out from the paper without overlap between the two. Figure 1 indicates the migration of the C and HMC bands on the paper with reference to the points of addition of the sample, and indicates the relative sizes of the spots.

In the next experiments a 20 λ sample of a mixture of the free bases C, T, A, and G, from the acid hydrolysis of a sample of thymus DNA was added to an electrophoresis paper by streaking a narrow band across the paper 10 cm. from one end. This was done in two strokes with air drying after each. A 20 λ sample of the HMC solution was then streaked on directly over the other sample in the same manner. Other papers were prepared in a manner similar to this and the papers were run in the electrophoresis apparatus at various voltages and for varying lengths of time.

Under the same conditions which produced best results previously in the C and HMC experiments, (1000 V. for 2 1/4 hours) it was found that not only were the T, G, and A band separated from the C and HMC bands, but they were also separated from each other. Figure 2 illustrates a paper which was run under these conditions.

The next experiments involved eluting the bases from the papers and checking on the recovery in terms of the amounts of bases added. Before this could be done, however, it was necessary to get UV absorption spectra of known quantities of C and HMC in the solution used to elute the bases from the papers.

Figure 1. Diagram of electrophoresis paper showing electrophoretic migration of cytosine (C) and hydroxymethylcytosine (HMC)

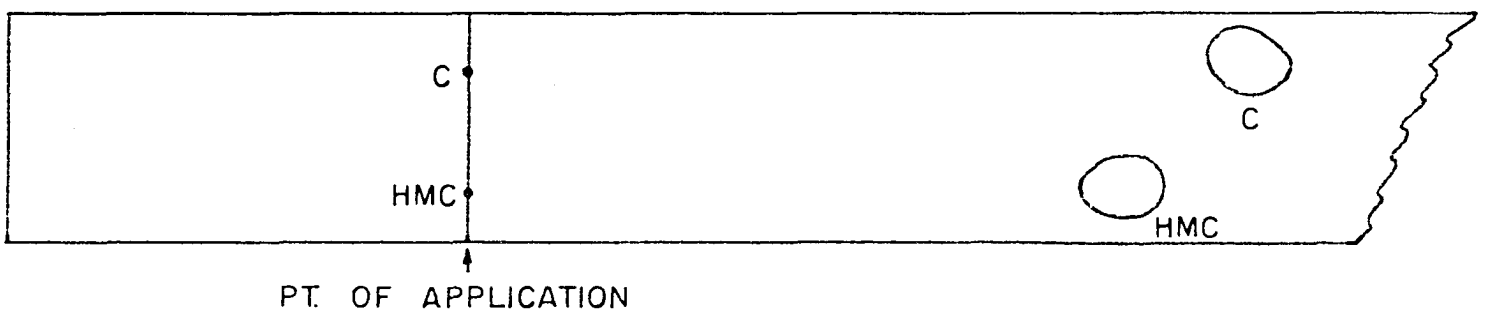
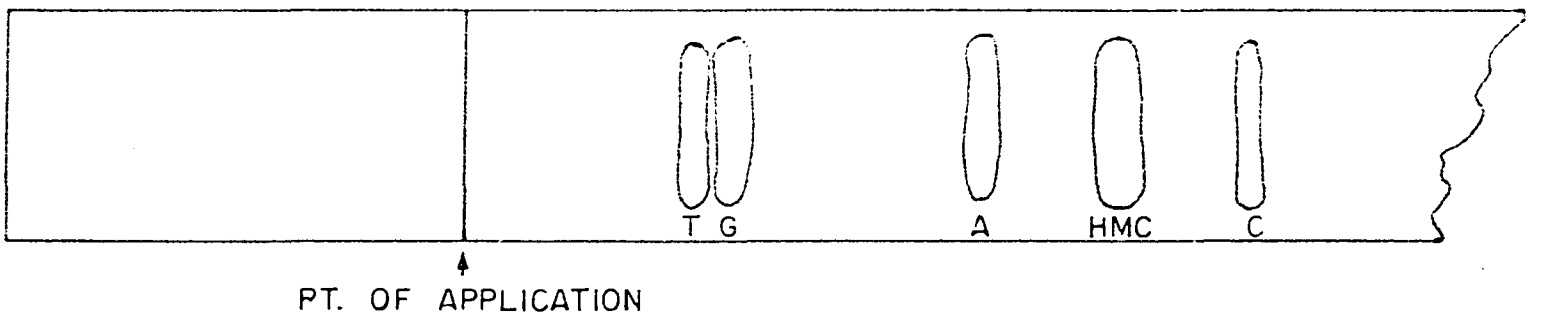

C
HMC
C
HMC
PT. OF APPLICATION

Figure 2. Diagram of electrophoresis paper showing electrophoretic migration of cytosine (C), hydroxymethylcytosine (HMC), thymine (T), adenine (A), and guanine (G)


T G
A
HMC
C
PT. OF APPLICATION

The solution used for elution of the bases from the papers was ammonium acetate buffer which was 0.1 M. in acetate at a pH of 4.3. Twenty λ samples of the C and HMC solutions mentioned previously were each added to 3.0 ml. quantities of this buffer in a pair of quartz cuvettes and the UV spectrum of each of these samples was read against the ammonium acetate buffer. The cell corrections were read at each wave length and subtracted from the previous readings to give corrected optical densities. Typical results are given in Table 1.

Table 1. Optical densities at various wavelengths for cytosine and hydroxymethyl cytosine

Wavelength in mμ	240	250	260	270	280	290	300
D for cytosine	.299	.399	.692	.944	.824	.367	.036
D for hydroxymethyl cytosine	.297	.279	.414	.589	.575	.319	.080
Highest density for C	.956 at 272 mμ						
Highest density for HMC	.615 at 274.5 mμ						

The D readings are for 20 λ samples of the indicated solutions added to 3.0 ml. of 0.1 M. ammonium acetate buffer at pH 4.3

In a manner similar to that just described with solutions of C and HMC of known concentration, the molecular extinction coefficients of the two bases at 260 mμ was found. For C, $\epsilon_{260} = 6200 \frac{1}{\text{mole cm.}}$ at pH 4.3, and for HMC, $\epsilon_{260} = 4460 \frac{1}{\text{mole cm.}}$ at the same pH.

In order to test for recovery of the bases from the paper after an electrophoresis run, 20 λ samples of C and HMC were streaked on papers in the manner indicated previously and run at 1000 V. for 2 1/4 hours. After the bands had been located by means of the UV lamp, they were cut out in rectangular pieces whose areas were measured. Samples of paper in rectangular pieces adjacent to the bands were cut for paper blanks. The papers were rolled up and placed in small test tubes and 3.0 mls. of 0.1 M. ammonium acetate buffer at H 4.3 was added to each tube. After the tubes had stood for 8 to 12 hours, the liquids were poured off into spectrophotometer cuvettes and the optical densities were read at several wave lengths against the same ammonium acetate buffer as used for eluting. The optical densities of the papers per square centimeter were determined. The C and HMC samples were then corrected on an area basis for absorption due to the paper alone. These readings were compared with readings similar to those in Table 1 for 20 λ samples of C and HMC added directly to 3.0 mls. of the 0.1 M. ammonium acetate buffer pH 4.3.

Results indicated recoveries in these experiments to be 90 - 95%. Similar experiments with HMC and an acid hydrolysate of thymus DNA showed HMC recoverable from the paper in this case to be approximately 85%. In working with the C and HMC bases, it was found useful to check some of the ratios of optical densities at different wave lengths. Those used particularly were the $\frac{280}{260}$, $\frac{270}{280}$, and $\frac{270}{260}$ ratios. Typical values of these ratios for the bases in 0.1 M. ammonium acetate buffer at a pH of 4.3 are given in Table 2.

Table 2. Optical density ratios for C and HMC

Optical density ratios (wavelengths in mμ.)	$\frac{D_{280}}{D_{260}}$	$\frac{D_{270}}{D_{280}}$	$\frac{D_{270}}{D_{260}}$
Cytosine	1.19	1.14	1.36
Hydroxymethyl Cytosine	1.39	1.03	1.41

The correction necessary due to the UV absorbing materials eluted from the blank paper has been indicated previously. This correction is adequate for the cases where the pure bases C and HMC are added to the papers. In cases where the acid hydrolysate of DNA is streaked on the electrophoresis paper and run under the established conditions of 1000 V. for 2 1/4 hours, there is some additional dirt which is eluted from the papers which is evident in regions between bands. This dirt decreases in amount from the point of application of the material in the direction of motion of the bases. It is necessary, therefore, to make corrections on the basis of a blank cut from the end of the paper beyond the C band which is then modified according to position of the band under consideration on the paper. The correction for C, therefore, is always less than that for HMC due to the fact that there is less dirt at this location than at the location of the HMC band. As a final check on the validity of the correction, the ratios of the corrected optical densities $\frac{D_{280}}{D_{260}}$, $\frac{D_{270}}{D_{280}}$, and $\frac{D_{270}}{D_{260}}$ are calculated and the values compared with those given in Table 2. In most cases, the agreement is very close. In a few instances,

however, the comparison indicates a larger correction to be necessary. There is bound to be some variation in the amount of dirt present in each of the samples streaked on the various papers.

The general method used in estimating the amounts of phage DNA and bacterial DNA per bacterial cell as a function of time after multiple infection of E. coli B. with UV irradiated or unirradiated T_2r+ will be outlined in the paragraphs which follow. Specific details which differ from one experiment to the next will be discussed in connection with the experiments themselves in a later section.

Procedure

(1) Duplicate 2000 ml. cultures of E. coli B. are grown with aeration in M-9 medium to a concentration of approximately 1×10^9 bacteria per ml. in 4 liter bottles at 37° C. The cultures are mixed and then reseparated into 2000 ml. amounts.

(2) The phage inoculum (irradiated or unirradiated) is added at zero time.

(3) 120 ml. samples of the infected cultures are withdrawn and transferred at specific times to 12 mls. of 50% (gms. per 100 mls.) cold T. C. A. plus 0.4 mls. of 1% serum albumin and chilled for at least 15 minutes. (The procedure is based on the methods of Hershey, Dixon, and Chase (2), Schneider (39), Schmidt and Thannhauser (40), and Schneider (41).

(4) From each flask in step 3, 5 ml. samples are withdrawn and centrifuged for 10 min. at 5,000 r.p.m.; the supernatants are poured off and discarded. To the precipitate is added 1 ml. of distilled water. These tubes are then used in determinations of total DNA (both bacterial and phage) by means of the Dische diphenylamine test.

(5) Each sample (127 ml. now) is divided into three parts containing approximately 42 ml. each and centrifuged for 10 min. at 5,000 r.p.m. Aliquots of each supernatant are kept for further check and the remainder of the supernatants, which contains the acid soluble fraction, are discarded. The 3 precipitates forming parts of the original sample withdrawn are washed into a single glass centrifuge tube with very small quantities of water. Five ml. of cold 0.3 M TCA are added and the acid insoluble material is reprecipitated with centrifugation under the same conditions as before. The supernatant is poured off and discarded.

(6) The precipitate is dissolved in 5 ml. of 1.0N. NaOH added to each tube and the tubes are heated for 18 hours at 37° C. This procedure depolymerizes any ribonucleic acid (RNA) present by alkaline hydrolysis.

(7) After the tubes have cooled in ice water, 0.833 mls. of cold 6N. HCL plus 0.59 ml. of cold 3.0 M. TCA are added to each tube. The tubes are allowed to stand in ice water for 15 minutes. the tubes are then centrifuged for 10 min. at 5,000 r.p.m. and

the supernatants poured off and discarded. Five ml. of 0.3 M. TCA is added to each precipitate, and the precipitates are re-precipitated under conditions similar to those mentioned previously. The supernatants are again poured off and discarded.

(8) To each precipitate is added 5 ml. of 0.3 M. TCA. The tubes are then heated at 90°C with frequent stirring, for 15 minutes. This process is supposed to dissolve out the DNA. Therefore after the tubes are cooled the tubes are centrifuged at 5,000 r.p.m. for 10 minutes and the supernatants are poured off into labelled tubes and saved. Five ml. of 0.3 M. TCA is added to the precipitate which, after stirring to wash, is reprecipitated with a repeat centrifugation. The supernatants are added to the previous corresponding supernatants. The precipitates, which consist primarily of protein material, are discarded.

(9) An aliquot of each of the above supernatants is withdrawn and used for a Dische diphenylamine estimation of the DNA present at this point. By comparing these figures with those gained in step 4 of the procedure a measure of recovery of total DNA up to this point is had.

(10) Each of the tubes containing solutions of DNA is heated for one hour at 100°C to help decompose the remaining TCA. After this, the drying apparatus, mentioned in one of the previous sections, is used to evaporate the material to dryness.

(11) Three ml. of 6N. HCl is added to each of the tubes to dissolve the dried material, and the tubes are heated for 3 hours at 100°C

in a boiling water bath. This hydrolyzes the depolymerized phage and bacterial DNA mixture to the five bases C, HMC, T, A, and G (2).

(12) The drying apparatus is used once again to evaporate the materials to dryness.

(13) One hundred λ's of 0.1N. HCl is used to dissolve the dried materials in each of the tubes.

(14) The entire sample from step 13 is added to an electrophoresis paper 10 cm. from one end by several streaks with a 25 λ micropipette. The paper is then wet with 0.1 M. tris formate buffer at pH 3.4 and placed in the electrophoresis apparatus containing the same buffer in its compartments.

(15) One thousand V. is applied across the paper for 2 1/4 hours. Actually 3 to 6 papers are run simultaneously.

(16) The papers are removed from the apparatus and air dried. The bands are located in a dark room with the aid of a portable UV lamp and the boundaries of the bands are marked lightly with a pencil.

(17) The distance from the origin to the center of each band is measured. The bands are cut out in rectangular pieces and each dimension is measured for a determination of the area. A rectangular strip is cut out also in a region just ahead of the band which is farthest from the origin. This piece, whose area is also determined, is used as a blank in determination of the UV absorption of the eluted bases.

(18) The papers are rolled up with forceps and placed in small test tubes. Three mls. of 0.1 M. ammonium acetate buffer at pH 4.3 is added to each of the tubes. These are left to stand overnight or for 8 hours.

(19) The solutions which contain the eluted bases, are then poured into spectrophotometer cuvettes and the optical densities are read in the spectrophotometer against the eluting buffer at wavelengths spaced 10 mμ. apart from 300 to 240 mμ. Cell corrections are subtracted from these readings. (In most instances, the optical densities of the T, A, and G bands were not read.)

(20) The optical density readings for C, and HMC are corrected for absorption due to blank paper and dirt by the method indicated previously.

(21) In order to get a measure of the concentration of the bases, C and HMC, the corrected optical density readings at 260 mμ are divided by the corresponding extinction coefficients x 10^{-4} at 260 mμ. After multiplying by the number of milliliters of eluate the data is in the form of moles of C and HMC per sample x 10^{7}.

(22) Since one-fourth of the bases in E. coli B. DNA is C whereas one-sixth of the bases in T_2r+ + DNA is HMC it is next necessary to multiply the final results in step 21 by 4 in the case of C and by 6 in the case of HMC in order to get a measure of the number of moles of DNA - P per sample x 10^{7} for the bacterial DNA and phage DNA.

(23) The moles of bacterial DNA - P x 10^7 from step 22 are now divided by 0.62 (this point will be discussed in a later section) and the total moles of DNA-P for a particular sample are arrived at by addition.

(24) Were it not for the fact that there are rather wide variations in the recoveries of total DNA for various samples in steps 5 to 8 as is obvious by a comparison of the results of the Dische diphenylamine test before and after these procedures, one could convert these values themselves to suitable units and plot them directly. Since the recoveries do vary considerably from sample to sample in this step and also in later procedures, it is preferable to find the fractions of the total DNA-P which are due to bacterial DNA-P and those which are due to phage DNA-P from steps 22 and 23. These are then multiplied by the original total moles of DNA-P as indicated by the diphenylamine test in step 4 and converted to the desired units for plotting against the time after infection that the sample was withdrawn.

The unit chosen in which to express quantity of DNA is that used by Hershey (2) which is phage units per bacterium. The amount of DNA equivalent to one phage unit must be determined. Since the standard used in the diphenylamine test for DNA was a secondary standard, a solution of calf thymus DNA, it was necessary to standardize it against a primary standard, a solution of T_4r+ phage DNA of known phosphorus content, by means of the diphenylamine test. The optical densities at 600 mμ read in the experiments in this test are then easily converted to moles of

DNA-P per sample. At the same time the secondary standard was compared with the primary standard in the diphenylamine test for DNA, a comparison was also made by the diphenylamine test on the acid insoluble fraction of a T_2r+ phage stock which had been assayed for active phage. In this way the amount of DNA-P per phage was determined. There are two factors which make this figure difficult to determine accurately: first, the assay procedure for the phage is subject to the usual statistical probable error and yields somewhat different values for each assay even of the same stock, and second, some of the phage are inactivated in the cleaning of the phage in the preparation of stocks which would tend to make the apparent amount of DNA-P per phage particle too high. An estimation of this inactivation may be found by comparing the DNA content of the phage suspension and its assay at an earlier step in the phage cleaning procedure with these quantities in the final phage stock.

The value for one T_2r+ phage unit is estimated to be $2.3 \times 10\text{-}11$ μg DNA-P, for the particular strain of T_2r+ used in these experiments, after several estimations with the preceding factors taken into consideration. This conversion factor is used in expressing the amount of phage DNA and the amount of bacterial DNA (step 24) and is eventually plotted against time.

EXPERIMENTS WITH UV IRRADIATED AND UNIRRADIATED BACTERIOPHAGE

Early Experiments

In the early part of this work, one object of the experiments was to endeavor to determine, if possible, the effect of ultraviolet light upon the bacteriophage causing it to become inactivated. Cohen and Arbogast (42) had performed infection experiments with irradiated phage and had found an apparent block in the synthesis of DNA in E. coli B. infected with a multiple infection of UV irradiated T_2r+. This indicated that a good starting point might be to isolate and study the acid soluble UV absorbing materials which, according to Cohen, continued to pile up even though the DNA synthesis was blocked for periods of time up to 45 minutes after infection.

In attempting to perform experiments similar to those of Cohen, three modifications were made in his procedure. First, infection should be accomplished in a medium with a limited energy source. The source of energy could then be added to start things off all at once. This would synchronize better the synthesis of phage materials in the cells. It would also make it more justifiable to assume that the average number of phage particles actually infecting simultaneously a bacterium would be equal to the phage to bacteria ratio. Second, the irradiation with ultraviolet light should be sufficient to inactivate the phage to a survival low enough that the fraction of bacteria infected with at least one active phage

particle would be very small. This fraction should be small enough so that any contribution to DNA synthesis by these bacteria could be neglected.

The third modification was to use T_4r+ (actually the tryptophane requiring mutant $T_{4.38}r+$) which had been shown in previous experiments in our laboratory (43) to be biochemically somewhat simpler than T_2r+ in the respect that each HMC, rather than only part of the HMC's, in the phage nucleic acid has a glucose molecule attached, probably in the hydroxymethyl group.

One method used regularly for starting processes simultaneously after adsorption of the phage by the bacteria is to infect the bacteria with the phage in buffer solution and then to add to this an equal volume of twice concentrated nutrient materials at zero time. The bacteria are first grown in nutrient medium, precipitated by centrifugation, and resuspended in buffer preparatory to the adsorption process. M-9 medium is essentially a buffer with glucose added as its only energy source. With M-9 medium, it is possible, then, to add glucose after infecting in the medium M-9 without glucose in order to start phage synthesis. By growing the bacteria originally in a medium which is essentially M-9 except that it has only a limiting amount of glucose, the cells exhaust the glucose supply and grow only to a limited concentration. After this concentration remains constant for an hour or so, the medium can be considered to be essentially glucose free and the bacteria may be infected with phage. The glucose may then be added to start the processes.

The addition of 15.5 ml. of sterile 10% glucose to a 2 liter quantity (or approximately 0.08% glucose) of M-9 without glucose was found to allow E. coli B. to grow up to a concentration of about 1×10^9 cells per ml. This quantity of bacteria would make biochemical studies feasible. It was convenient to calibrate the spectrophotometer to read bacterial concentrations under these conditions. Samples were drawn from the culture at various times after the inoculum had been added. An aliquot of each was serially diluted and plated for bacterial colony count while the remainder was placed in a spectrophotometer cuvette and the optical density read at 500 mμ. In this way the spectrophotometer was calibrated so that the progress of the increase of bacterial concentration might be followed by periodically checking the optical density of the solution at 500 mμ. In Table 3 a rough calibration for the spectrophotometer in terms of bacterial concentration is given for M-9.

Table 3. Calibration of spectrophotometer for bacterial concentrations

Concentration of E. coli B. (bacteria per ml.)	D_{500}
7.5×10^7	0.048
9.4×10^7	0.064
1.63×10^8	0.105
2.2×10^8	0.183
5.6×10^8	0.354
1.2×10^9	0.540
1.52×10^9	0.642

The optical density at 500 mμ of the M-9 medium alone is 0.003.

Experiments with unirradiated phage

Cohen's experiments, with the above mentioned modifications, were carried out with unirradiated T_4r+. The bacteria were grown in M-9 with limiting glucose to a concentration of approximately 1×10^9 bacteria per ml. where the concentration remained constant for about an hour before addition of the phage. The phage to bacteria ratios used were from 3 to 7. After allowing 10 minutes for phage adsorption sterile 10% glucose was added to a final concentration of 0.4%. The time of addition of the glucose was indicated as zero time. Samples of the culture were withdrawn periodically. Aliquots of these samples were used for the diphenylamine test for total DNA while other aliquots were diluted and the optical densities read at 260 mμ.

In general, the results of these experiments resembled those of Cohen; the UV absorption increased with time and the total DNA increased with a short lag period at the beginning. Some difficulty was encountered in measuring the UV absorption of the samples. The concentration was too great to permit direct reading. When a dilution was made the deviation of the points from the best smooth curve drawn through the points was very great. A large fraction of the apparent absorption was actually due to the radiation scattered out of the beam by the particles suspended in the diluted culture. This was clearly shown by a comparison of the apparent absorptions in buffer read against buffer with those for a dilution in glycerol read against glycerol. In the glycerol, where the index of refraction is higher, the apparent absorptions were much lower than the

corresponding readings in buffer. The fact that part of the apparent absorption is due to scattering is also indicated by the fact that when part of the cells lyse at the end of the first and second latent periods the apparent absorption decreases. At these times the total UV absorbing substance is not decreasing whereas the scattering is decreasing due to the decrease in the number of the cells. The UV absorption measurements were not in any event, completely satisfactory.

Experiments with irradiated phage

Several experiments similar to those just described were performed with UV irradiated phage. Phage with survivals from 0.03 to 0.003 were used. The total DNA, in each experiment, increased very little with time with the rate of increase approximately proportional to the phage survival. The UV absorption measurements followed the same general trend but were beset with the same difficulties as those in the case of the unirradiated phage. When the cultures were allowed to remain at $37^{\circ}C$ under aeration for 12 to 16 hours and an assay of the phage was made, the phage yield per bacterium was found to be very low.

Discussion

The results listed in the previous section were different than those of Cohen in similar experiments. They seemed to indicate that if multiplicity reactivation was taking place at all, it was taking place in a very small fraction of the cells. Experiments designed specifically to check for multiplicity reactivation under the conditions used in the

previous experiments were in order and were next performed. Along with these, experiments known to give multiplicity reactivation were set up for comparison, and several parameters were varied in an attempt to determine the conditions necessary for multiplicity reactivation to take place.

Tests for Multiplicity Reactivation

Theory

When a bacterial suspension is infected with phage at a given phage to bacteria ratio the distribution of the phage among the bacteria may be estimated by a Poisson distribution. In this way the fraction of the cells containing no adsorbed phage, those containing one adsorbed phage, those containing two adsorbed phage, and so on, may be approximated. The general expression for the fraction of the bacteria having n phage when the phage to bacteria ratio is given by m is:

$$P_n = \frac{m^n e^{-m}}{n!} .$$

The fraction of bacteria having no adsorbed phage will therefore be $P_o = e^{-m}$; that having one adsorbed phage will be me^{-m}, etc.

Since a bacteria must have at least one phage adsorbed in order to give rise to a plaque, the fraction of bacteria able to give rise to plaques will be $1-e^{-m}$. If the phage are irradiated with ultraviolet light to a survival of S^* and these irradiated phage are used to infect cells at

*The survival is based on the fraction able to give rise to plaques when bacteria are singly infected with the irradiated phage.

a multiplicity of m, the effective multiplicity will now be the product mS. In the event that no bacterium gives rise to a plaque which does not have at least one active phage particle adsorbed to it, the fraction of the bacteria giving rise to plaques will be given by the expression: $P_s = 1-e^{-mS}$. As the product mS approaches zero this fraction approaches mS, and the curve of $1-e^{-mS}$ plotted against m, for values of S very low compared to m, approaches a straight line.

As mentioned in the Introduction, Luria (6) discovered that when E. coli B. cells are infected with UV irradiated phage T_2, T_4, or T_6 (the T even phages) at phage to bacteria ratios greater than one, the fraction of bacteria giving rise to plaques is much greater than $1-e^{-mS}$. This effect was studied by Luria and Dulbecco (15) and called multiplicity reactivation. For phage irradiated to survivals in the range of 10^{-2} to 10^{-3} (which are used in this set of experiments) the fraction of the cells giving rise to plaques approaches the fraction of the cells which have two or more UV irradiated phage adsorbed. Assuming again a Poisson distribution of phage among the cells this fraction equals $1-e^{-m}-me^{-m}$ where the second and third terms of this expression are the fraction of cells with zero and one phage adsorbed respectively.

By comparing the fraction of the bacteria giving rise to plaques, in the various cases, with the product mS on the one hand and $1-e^{-m}-me^{-m}$ on the other, a check on whether or not any multiplicity reactivation takes place in each case may be made.

Experimental

E. coli B. cells were grown in M-9 with limiting glucose to a concentration of approximately 1×10^9 bacteria per ml. and then diluted to a concentration of $1 \times 10^8 \frac{\text{bacteria}}{\text{ml.}}$ with phage buffer. The concentration was checked by colony count. Phage $T_{4.38}r+$, irradiated to a survival in the neighborhood of 0.01, were added at various phage to bacteria ratios, to aliquots of the bacterial suspension and ten minutes was allowed for adsorption to take place at 37°C. At the end of this period 0.1 ml. was removed from the adsorption tube and transferred to 9.9 mls. of cold phage buffer. From this first dilution tube an aliquot was removed to check for the amount of phage adsorption which had actually taken place. Another 0.1 ml. aliquot from this tube was transferred to 9.9 mls. of cold phage buffer in the second dilution tube and the remainder of the serial dilution carried out. Samples were finally plated to determine the fraction of bacteria giving rise to plaques.

One of two methods was used to determine the actual amount of phage adsorption which had taken place in the ten minute adsorption period. The first method was a direct method. The aliquot removed from the first dilution tube was centrifuged at a speed sufficient to spin out the bacteria. The supernatant was then assayed for remaining free phage (after correction for survival) and the multiplicity of adsorption corrected accordingly.

The first method was found subject to considerable error in instances where a small fraction of the bacteria remained in the supernatant after centrifugation as was found to be true in using the small clinical centrifuge.

The error was greater the lower the survival values and the higher the values of m. This was because the probability of these bacteria giving rise to plaques was by no means negligible in comparison with the plaques due to free phage where less than one in a hundred phage could give rise to a plaque. The second method was less subject to error under the conditions mentioned. In this case a colony count of bacteria was made after serial dilution and plating of the aliquot removed from the first dilution tube. Since a bacterial cell is killed by the adsorption of one or more phage (irradiated or nonirradiated) and cannot give rise to a colony when plated, the fraction of bacteria still giving rise to colonies represent the fraction having no adsorbed phage. If one again assumes a Poisson distribution it is possible to determine the actual m of adsorbed phage since the fraction of bacteria with no phage adsorbed may be given by e^{-m}.

After the experiment had been performed a few times with cells grown in M-9 with limiting glucose, it was performed with cells grown in M-9 (without limiting glucose), and with cells grown in tryptone broth. The cells grown in regular M-9 or tryptone broth were centrifuged out, taken up in phage buffer, centrifuged out once again, and resuspended in phage buffer for adsorption. All of these experiments were done using UV irradiated $T_{4.38}r+$. Similar tests for multiplicity reactivation were then run for bacteria grown under these various conditions but infected with UV irradiated T_2r+.

Results

Typical results of the tests for multiplicity reactivation using $T_{4.38}r+$ are listed in Table 4. Similar results for the remainder of the experiments are listed in a more abbreviated form in Table 5.

The results listed in Table 4 indicate that there was no multiplicity reactivation for the UV irradiated $T_{4.38}r+$ phage on E. coli B. cells grown in M-9 with limiting glucose, whereas for E. coli B. cells grown in regular M-9 which were washed and resuspended in phage buffer, there was a small amount of multiplicity reactivation. When broth grown cells which had been washed and resuspended in phage buffer were used the results for $T_{4.38}r+$ were similar to those for cells grown in M-9 indicating that this particular tryptophane requiring mutant does not give multiplicity reactivation under the same conditions as does ordinary T_4r+.

Results of experiments listed in Table 5 show that T_2r+ does not give multiplicity reactivation in cells grown in M-9 with limiting glucose. More reactivation takes place with cells grown in M-9 than in the corresponding case with $T_{4.38}r+$. For cells grown in tryptone broth, the results clearly indicate that multiplicity reactivation takes place with T_2r+.

When a low concentration of Ca^{++} ion was added to the M-9 medium in which the cells were grown, some increase in the apparent multiplicity reactivation with T_2r+ was noted. The effect was much greater in cases where nutrient materials containing amino acids, vitamins, and minerals were added to the M-9 in which the E. coli B. bacterial cells were grown. This was in the form of small quantities of yeast extract and

Table 4. Results of multiplicity r

General growth conditions for E. coli B. Phage type and survival	(A) Concentration of bacteria at adsorption	(B) Concentration of phage at adsorption	(C) Plaques formed per ml.	(D) Phage to bacteria ratio (m)	(E) Percent adsorption	(F) Actual m adsorbed phage
1) M_9 with limiting glucose / $T_{4.38}^{r+}$ S= 0.010	5.15 x 10^7 / ml.	5.35 x 10^7	2.8 x 10^5	1.04	75	0.78
		1.07 x 10^8	4.4 x 10^5	2.08	75	1.56
		2.14 x 10^8	1.32 x 10^6	4.16	75	3.12
2) M_9 / $T_{4.38}^{r+}$ S = 0.010 / Adsorption in buffer	1.04 x 10^8 / ml.	5.35 x 10^7	6.7 x 10^5	0.515	94	0.484
		1.07 x 10^8	1.75 x 10^6	1.03	90	0.926
		2.14 x 10^8	7.6 x 10^6	2.06	90	1.852
		4.28 x 10^8	9.6 x 10^6	4.12	90	3.7
3) Tryptone broth / $T_{4.38}^{r+}$ S = 0.015 / Adsorption in buffer	2.5 x 10^7 / ml.	6.7 x 10^7	7.5 x 10^5	2.68	40	1.07
		1.34 x 10^8	2.4 x 10^6	5.36	50	2.68
		2.68 x 10^8	5.2 x 10^6	10.72	47.5	5.1
		5.35 x 10^8	11.0 x 10^6	21.44	50	10.72

Table 4. Results of multiplicity reactivation tests

es d l.	(D) Phage to bacteria ratio (m)	(E) Percent adsorp-tion	(F) Actual m of adsorbed phage	(G) Calculated value of mS	(H) Calculated value of $1-e^{-m}-me^{-m}$	(I) Experimental determination of plaques to bacteria ratio	(I)/(G)
10^5	1.04	75	0.78	0.078	0.185	0.00544	0.7
10^5	2.08	75	1.56	0.0156	0.46	0.00854	0.54
10^6	4.16	75	3.12	0.0312	0.82	0.0256	0.82
10^5	0.515	94	0.484	0.0048	0.083	0.00644	1.34
10^6	1.03	90	0.926	0.0093	0.237	0.0168	1.81
10^6	2.06	90	1.852	0.0185	0.552	0.073	3.95
10^6	4.12	90	3.7	0.037	0.883	0.0923	2.5
10^5	2.68	40	1.07	0.016	0.296	0.03	1.88
10^6	5.36	50	2.68	0.04	0.75	0.096	2.4
10^6	10.72	47.5	5.1	0.0765	0.964	0.208	2.72
10^6	21.44	50	10.72	0.16	0.999+	0.44	2.75

Table 5. Results of further multiplicity rea

Expt.	Phage type	Survival	General growth conditions for E coli B	Actual m of adsorbed phage	Calculated value of mS	C 1
1)	T_2r+	0.019	Tryptone broth	1.2	0.0228	
2) a.	T_2r+	0.007	Tryptone broth	1.94 3.86 7.76	0.0136 0.027 0.054	
	T_2r+	0.007	M_9	0.78 1.56 3.10	0.00540 0.0110 0.0226	
c.	T_2r+	0.007	M_9 with limiting glucose	1.67 3.34	0.0116 0.0234	
d.	T_2r+	0.007	M_9 with limiting glucose. Cells washed and resuspended in buffer for adsorption	1.9 3.8	0.0132 0.0274	
3) a.	T_2r+	0.0059	M_9	1.63 3.24	0.0086 0.0192	
b.	T_2r+	0.0059	M_9 with 3.0 x 10-4 M. $CaCl_2$	2.13 4.25	0.0126 0.0251	
4)	T_2r+	0.0039	M_9 containing 0.165 gms. Yeast extract per liter plus 2.2 gms. Casamino acids per liter + 10-4 M. $CaCl_2$	0.66 1.33 2.66	0.00258 0.0052 0.0104	

Table 5. Results of further multiplicity reactivation tests

General growth conditions for E coli B	Actual m of adsorbed phage	Calculated value of mS	Calculated value of $1-e^{-m}-me^{-m}$	Experimental determination of plaque to bacteria ratio
Tryptone broth	1.2	0.0228	0.338	0.282
Tryptone broth	1.94	0.0136	0.577	0.35
	3.86	0.027	0.9	0.54
	7.76	0.054	0.98	0.63
M_9	0.78	0.00540	0.185	0.037
	1.56	0.0110	0.46	0.071
	3.10	0.0226	0.82	0.112
M_9 with limiting glucose	1.67	0.0116	0.49	0.0052
	3.34	0.0234	0.85	0.0107
M_9 with limiting glucose. Cells washed and resuspended in buffer for adsorption	1.9	0.0132	0.74	0.0122
	3.8	0.0274	0.93	0.0218
M_9	1.63	0.0086	0.48	0.070
	3.24	0.0192	0.84	0.098
M_9 with 3.0×10^{-4} M. $CaCl_2$	2.13	0.0126	0.63	0.170
	4.25	0.0251	0.94	0.208
M_9 containing 0.165 gms. Yeast extract per liter plus 2.2 gms. Casamino acids per liter + 10^{-4} M. $CaCl_2$	0.66	0.00258	0.13	0.15
	1.33	0.0052	0.39	0.42
	2.66	0.0104	0.75	0.50

casamino acids added when the medium was being made up. The apparent multiplicity reactivation resulting in this case with T_2r+ was as great as that for T_2r+ in cells grown in tryptone broth.

Leakage and "Lysis-From-Without"

In order to discover whether this apparent multiplicity reactivation, which seemed dependent on certain materials in which the cells had been grown, was actually dependent on multiple infection, single infection experiments with T_2r+ on cells grown in these various media were performed. Cells were grown in the various media, were washed and resuspended in phage buffer, and singly infected with T_2r+. Adsorption was allowed to take place for 20 minutes in phage buffer at 37°C. Results of these experiments indicated that even in single infection there were 6 to 8 times less plaques in the case of cells grown in M-9 than for broth grown cells. The M-9 plus Ca^{++} grown cells again gave 2 to 3 times as many plaques as M-9 grown cells. This suggested that the apparent differences in multiplicity reactivation experiments with T_2r+ did not have anything to do with multiple infection but were characteristic of T_2r+ infection in general.

In one experiment in which cells grown in M-9 were washed and resuspended in phage buffer and then infected with T_2r+ at a phage to bacteria ratio of approximately five, a decrease in the apparent turbidity of the culture and foaming took place. This was with no glucose present. Experiments were designed on a smaller scale to study this effect. An aliquot of each culture was removed before infection and one several

minutes after infection and the optical densities of these samples were compared at 500 mμ. The optical densities after infection for various phage to bacteria ratios were found to be from 0.35 to 0.6 times those previous to infection. The cells grown in M-9 + Ca^{++} seemed to give results very similar to those with cells grown in M-9 alone.

Discussion

Results in the experiment above indicate that probably "lysis-from-without" is occurring at much lower multiplicities than is ordinarily considered necessary in order for the phenomenon to occur. "Lysis-from-without" is the name given to the effect of lysis of cells without phage growth which usually occurs when a large number of phage particles are adsorbed to a bacterium. Benzer et al. (7) have noted, however, that lysis from without occurs at low multiplicities when a suitable energy source is absent.

The results of the single infection experiments with T_2r+ might indicate that E. coli B. cells which are grown in M-9 and washed and resuspended in phage buffer are much more fragile than broth grown cells submitted to similar treatment and than cells grown in M-9 with nutritional ingredients such as casamino acids or yeast extract added. Puck and Lee (44, 45) have studied leakage of cellular constituents produced by an increase in host cell permeability induced by bacteriophage infection. They suggest that in a normal infection process this leakage drops off in two or three minutes after infection due to a sealing reaction which closes up the hole (45) produced by the virus for the injection

of its DNA and starts another reaction which eventually renders the entire cell wall refractory to a further lytic stimulus of the same kind. The suggestion is also made that the processes which normally check the leakage might themselves be inhibited by the attachment of an overwhelming dose of virus particles to the same cell ("lysis-from-without") or nutrient deprivation (44, 46) (unchecked leakage).

The effect of the Ca^{++} with the M-9 may be to decrease the amount of leakage and thus to keep some of the cells alive to continue the phage production process. Puck and Lee (44, 45) found Mg^{++} to suppress leakage reaction in normal infection and suggest that other divalent ions may have the same effect. According to these workers, under the appropriate conditions, it appears that the leakage process merges into that called "lysis-from-without" as the hole size increases. The results of the experiments mentioned in the previous section do not contradict these hypotheses.

Changes in Amount of Total DNA with Time

Results of experiments mentioned in the preceding section, together with other experiments, indicated the desirability of performing experiments on the changes in amount of total DNA with time after infection with UV irradiated T_2r+, and with unirradiated T_2r+ as a control in systems where multiplicity reactivation is known to take place. Two workable methods, each with its own advantages and disadvantages, have been used. In one case the E. coli B. bacterial cells are grown in M-9 supplemented with small amounts of casamino acids, yeast extract, and

$CaCl_2$. The cells are washed by centrifugation and resuspended in a buffer consisting of M-9 medium without glucose. Adsorption of the T_2r+ phage is allowed to take place in this medium. At zero time an equal volume of M-9 containing double concentrations of glucose and the other nutrient ingredients is added to the mixture to make the medium the same as the medium in which the cells were grown and to enable the biosynthetic mechanisms to start operating. Samples are withdrawn at zero time and periodically thereafter as the virus particles are synthesized.

In another case the bacteria are grown in M-9 medium to which no supplementary ingredients have been added. The cells are not precipitated out with centrifugation, but rather, when the bacterial concentration reaches a proper value, the phage are added; this is zero time. The bacterial concentration is determined by colony count. Samples are again drawn at zero time and periodically thereafter.

The main advantage of the first method is that the biosynthetic process is begun at the same time in each cell. Since adsorption takes place in the buffer, the variability in the times of infection of the individual cells is not reflected in the times of beginning synthesis. In the second method this is not the case. Its principal advantage is, however, that the medium used is a well defined synthetic medium. Also, the time necessary to carry out the experiment is less in the second case since the centrifugation steps are omitted.

Examples of experiments performed by each of the two methods follow.

Experiment A - Method 1

E. coli B. bacteria were grown in duplicate 500 ml. Erlenmeyer flasks containing 250 ml. of supplemented M-9 medium to a concentration estimated to be approximately 1×10^9 cells per ml. The cells were precipitated by centrifugation, washed, and resuspended in a total value of 250 ml. of M-9 buffer (without glucose). After mixing thoroughly, 125 ml. was returned to each of the two aerating flasks which had been kept free from contamination. To one of these an aliquot of UV irradiated T_2r+ phage was added to give a final phage to bacteria ratio of approximately 3. Adsorption was allowed to take place for 10 minutes at 37^oC with aeration. To the other flask an identical aliquot of unirradiated phage was added. The phage were at the same concentration in each case, having been part of the same stock, with the only difference being that one was irradiated while the other was not. The adsorption conditions for the second flask were identical to those for the first.

At the end of the adsorption period 125 ml. of double concentration start-up mixture was added to each of the two flasks. This constituted zero time. Five ml. samples were withdrawn from each flask periodically from this point over a period of time Each of these samples was added immediately to 0.5 ml. of 3 M. TCA in tubes standing in ice water. The time (after zero time) of adding each 5 ml. sample to the TCA was noted and indicated as the time for that sample in graphs where the concentration of a particular material was plotted against time. In this case the material was total DNA which contained both bacterial and

phage DNA's.

After at least 10 minutes had been allowed for precipitation of the acid insoluble materials in the samples, the precipitates were centrifuged down. The supernatants were transferred to numbered tubes for use in another connection, and 1 ml. of cold distilled water was added to each of the precipitates. The relative amounts of total DNA present in each of the precipitates was found by means of the Dische diphenylamine test.

Experiment B - Method 2

The second method differed from the first only in the following ways. The bacteria were grown in duplicate flasks each containing 250 mls. of M-9 medium. When the concentration of bacteria reached the proper value, the aeration was stopped, the bacterial suspensions mixed thoroughly, and half returned to each flask again. The concentration was checked by colony count on an aliquot removed after the two batches had been mixed. The phage were added at zero time, and samples were withdrawn and tested as indicated in the first method.

General conditions

In each method the actual multiplicity of the adsorbed phage was checked by making a colony count of surviving bacteria on an aliquot removed a certain number of minutes after the phage had been added.

Other experiments using each of these methods were run, some employing different sized aeration flasks containing different amounts of medium, but the results in each case were similar to those for one of the two experiments described here.

The pertinent information connected with these two total DNA experiments (Experiment A and Experiment B) is given in Table 6.

Table 6. Comparison of Experiments A and B

Experimental conditions and results	Experiment A	Experiment B
Medium used for growth of E. coli B.	M-9 + nutrient ingredients	M-9
Adsorption of T_2r+	In buffer	Not in buffer
Concentration of bacteria during viral synthesis	1.16×10^9/ml.	7×10^8/ml.
Phage survival after UV irradiation	0.003	0.0075
Actual multiplicity (m) of adsorbed phage	2.5	3.0
Final concentration of T_2r+ (yield)		
Unirradiated case-----	1.2×10^{11}/ml.	7.2×10^{10}/ml.
Irradiated case ------	7×10^{10}/ml.	3.3×10^{10}/ml.
Final yield - phage per cell		
Unirradiated case-----	103	103
Irradiated case -------	60	47

Results

The results of the Dische diphenylamine test on the samples in Experiment A are plotted against time and shown in Figure 3. Those for Experiment B are plotted in Figure 4. Since the interest in these experiments is in relative amounts of DNA rather than in absolute quantities, and in a comparison of the irradiated phage case with the unirradiated case, the data is plotted in terms of the optical density of the diphenylamine complex at 600 mμ which is directly proportional to the total DNA per sample.

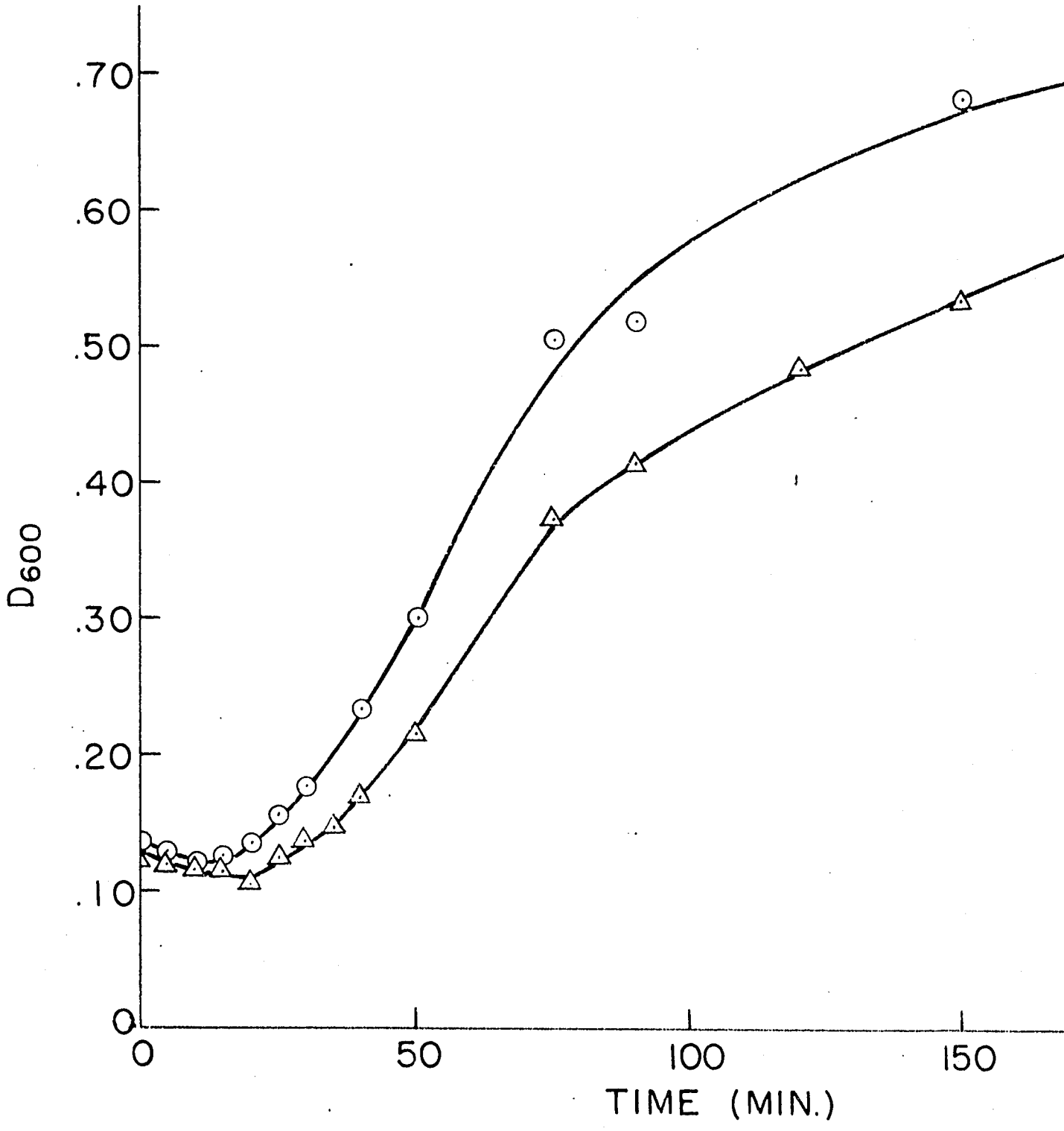
Figure 3. Dische reaction (D_{600}) of trichloroacetic acid insoluble fraction at various times after infection

⊙ Infection with unirradiated phage.

△ Infection with irradiated phage (survival = 0.003).

Original concentration of bacteria = 1.16×10^9/ml.

Actual multiplicity of infection = 2.5.


.70
.60
.50
.40
.30
.20
.10
0
D600
0
50
100
150
TIME (MIN.)

57b

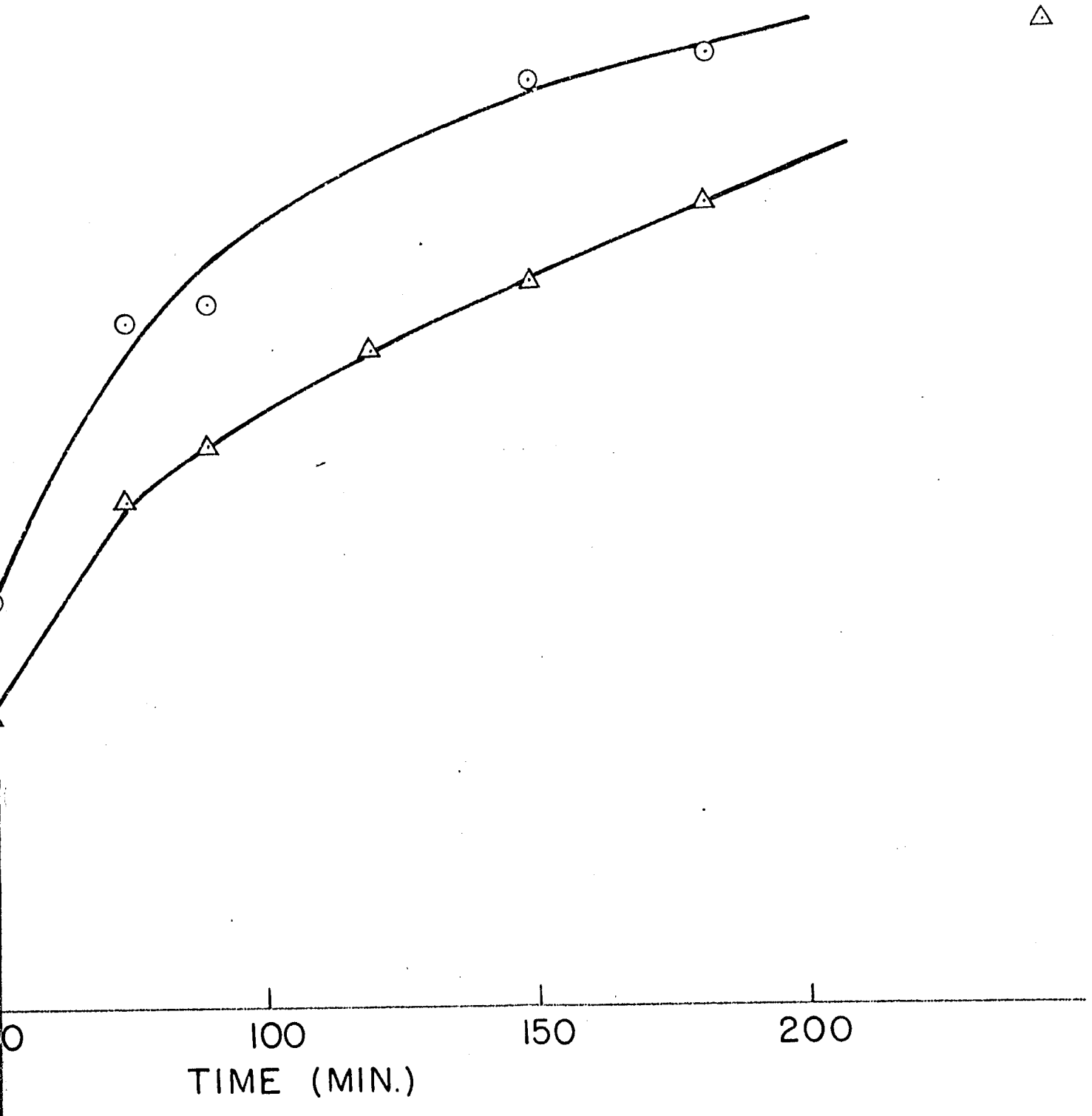


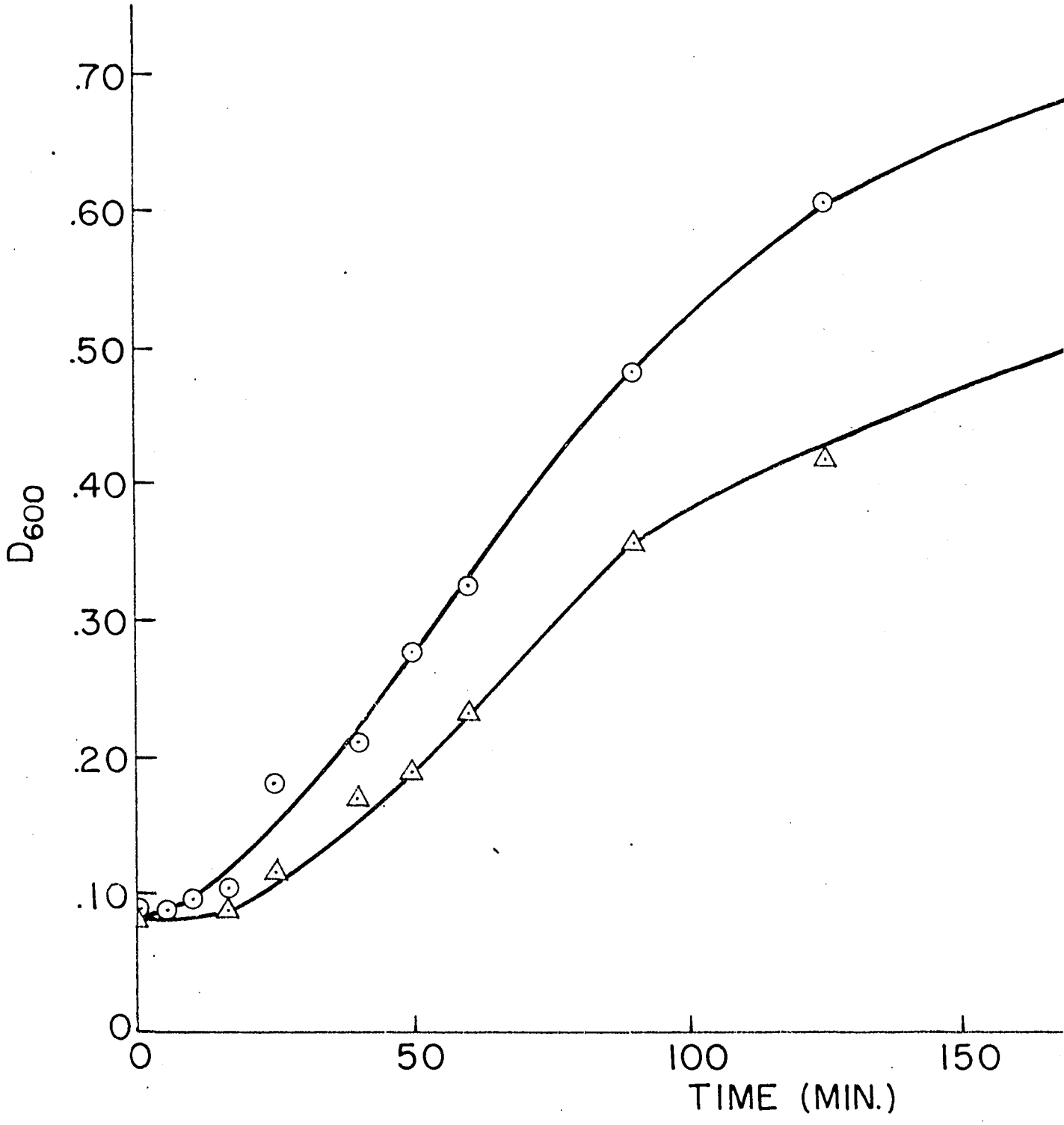
Figure 4. Dische reaction (D_{600}) of trichloroacetic acid insoluble fraction at various times after infection

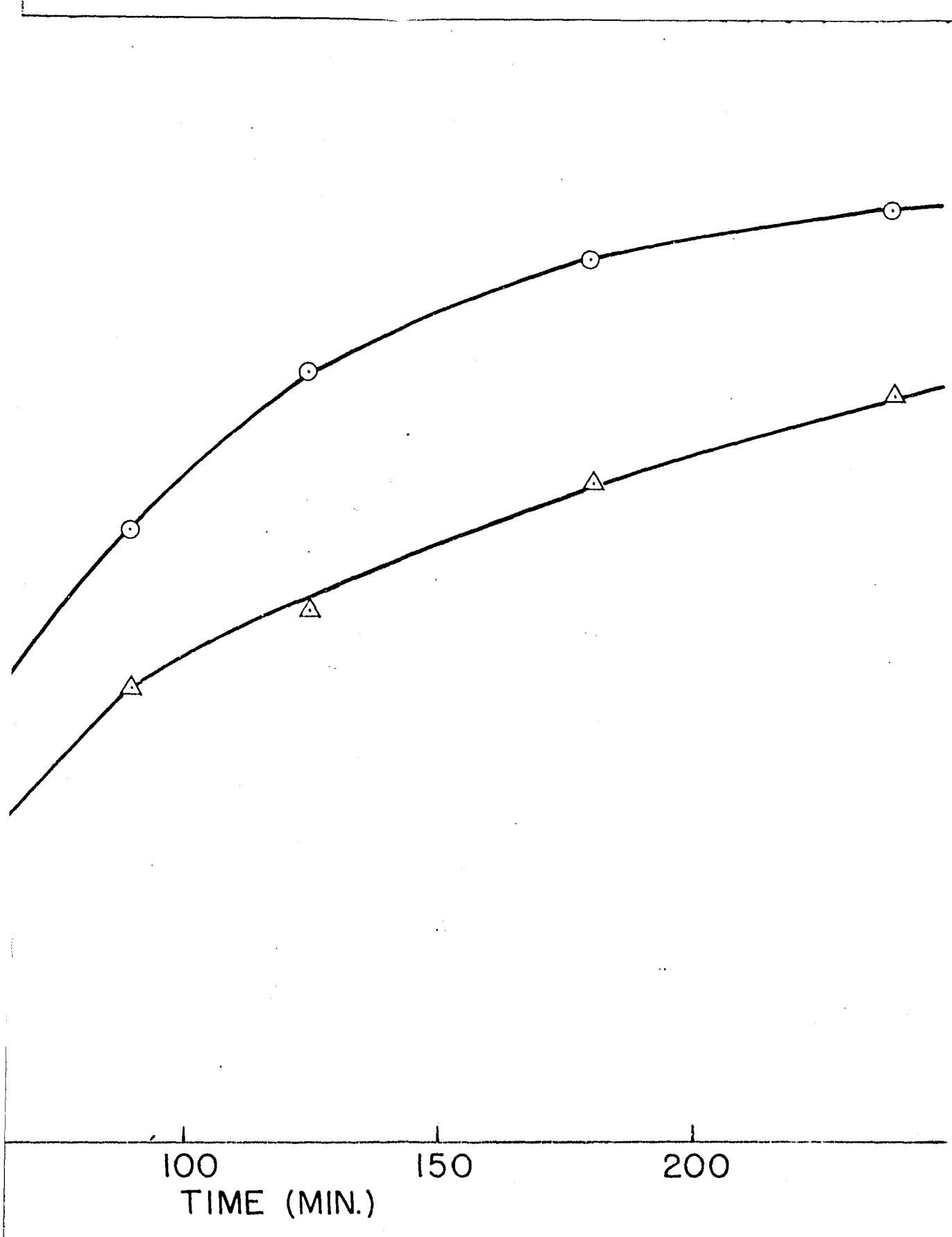
○ Infection with unirradiated phage.

△ Infection with irradiated phage (survival = 0.0075).

Original concentration of bacteria = 7×10^8/ml.

Actual multiplicity of infection = 3.0.


.70
.60
.50
.40
.30
.20
.10
0
D_{600}
0
50
100
150
TIME (MIN.)


100
150
200
TIME (MIN.)

Discussion

Figure 3 shows a slight drop in the total DNA with time for the first few minutes after infection with unirradiated phage, followed by a rapid increase which seems to begin to taper off at around 75 minutes. The curve representing infection with irradiated T_2r+ has the same general shape but lags the first curve by 8 to 10 minutes in the region of rapid increase in total DNA. The first curve also attains a higher value by the end of the period of measurement.

In general the curves in Figure 4 appear to be similar to those in Figure 3. The most significant difference is probably that there is no initial decrease in total DNA in either of the two curves but rather the DNA increases after a short lag period with the curve for infection with irradiated phage again lagging the first curve by approximately 8 to 10 minutes in the initial increase period. The initial decrease in total DNA in Experiment A may be due to an extra period of time necessary for adaptation to the medium before synthesis begins since the phage are adsorbed in buffer.

Changes in UV Absorbing Acid Soluble Material with Time

In connection with Experiment A and Experiment B of the last section, an estimation of the changes in UV absorbing acid soluble material with time was made. The supernatants of all withdrawn samples which, after precipitation in cold 0.3 M. TCA and centrifugation, had been poured into labelled tubes and stored in the cold room, were used for this purpose. Aliquots of 0.5 ml. from each tube were mixed with 2.5 ml. of

phage buffer in spectrophotometer cuvettes and the optical densities were read against buffer at 10 μ intervals between 240 and 300 mμ. The values were corrected for 0.3 M TCA blank values to get the optical densities of the acid soluble materials in the culture.

The corrected optical densities of the samples at 260 mμ have been plotted against the time after infection and are shown in Figures 5 and 6. Figure 5 represents data taken in connection with Experiment A where the cells were grown in supplemented M-9 medium, washed, resuspended, and infected in buffer. Figure 6 represents data from Experiment B where the cells were grown and infected in M-9.

In each of the two experiments the changes in UV absorbing acid soluble materials with time, for infection with both irradiated and unirradiated phage, appear to be the same from zero time up to approximately 60 minutes after which the amount of UV absorbing acid soluble materials in the case of irradiated phage is greater than in the case of unirradiated. The most apparent difference between the two experiments is that in the case where the cells are not washed and infected in buffer, there is an approximately steady increase in UV absorbing acid soluble material with time from zero as indicated in Figure 6. In the other experiment, the amount of these substances is constant for about 30 minutes after zero time, as indicated in Figure 5, and then increases rapidly changing gradually to a rate similar to that shown in Figure 6.

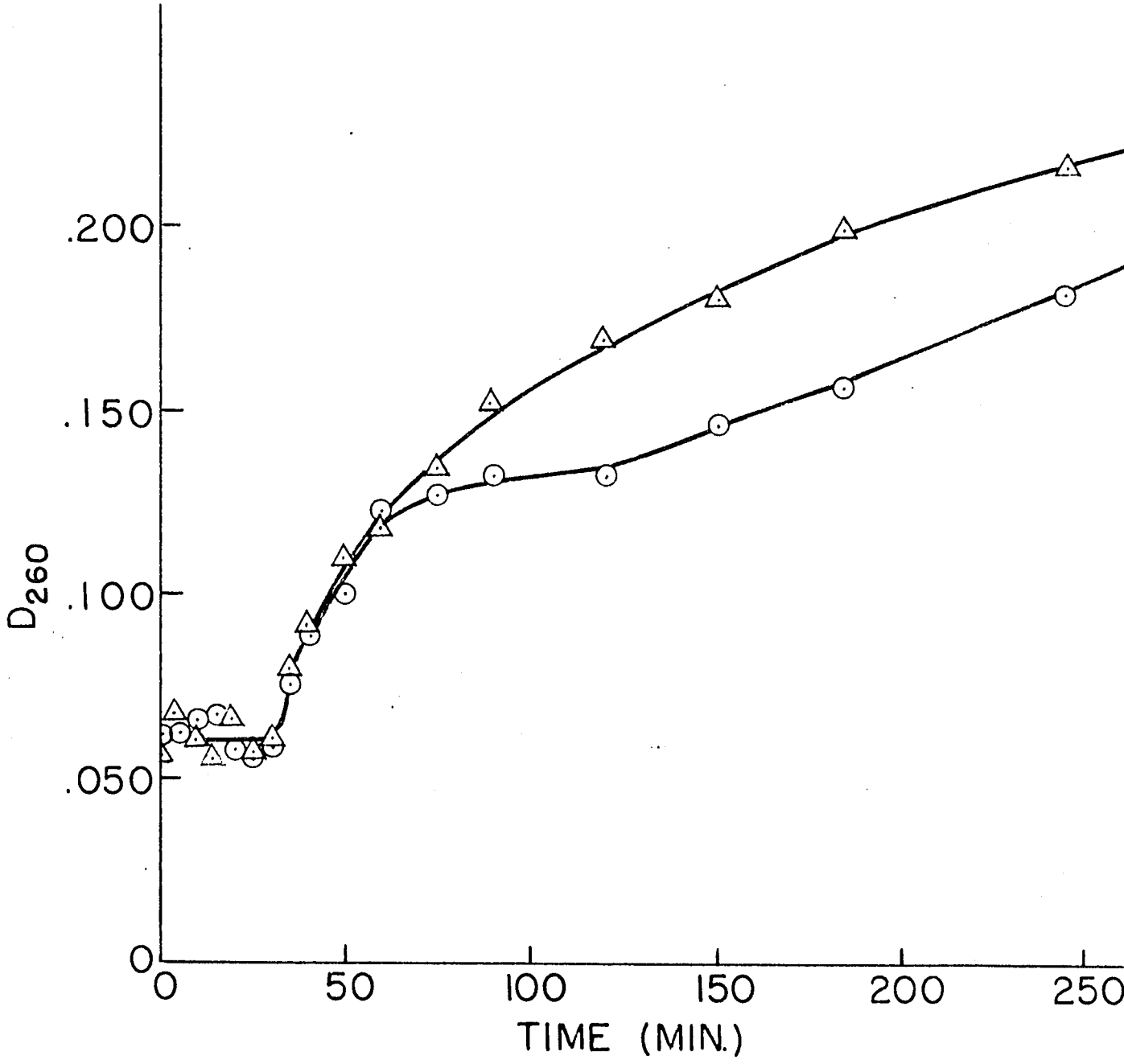
In Table 7 optical densities at several wavelengths at various times after infection together with differences in optical densities for the data plotted in Figure 5 are listed.

Figure 5. D_{260} of a 1/6 dilution of the trichloroacetic acid soluble fraction at various times after infection

⊙ Infection with unirradiated phage.

△ Infection with irradiated phage.

Conditions of infection as in Figure 3.


.200
.150
.100
.050
0
D_{260}
0
50
100
150
200
250
TIME (MIN.)

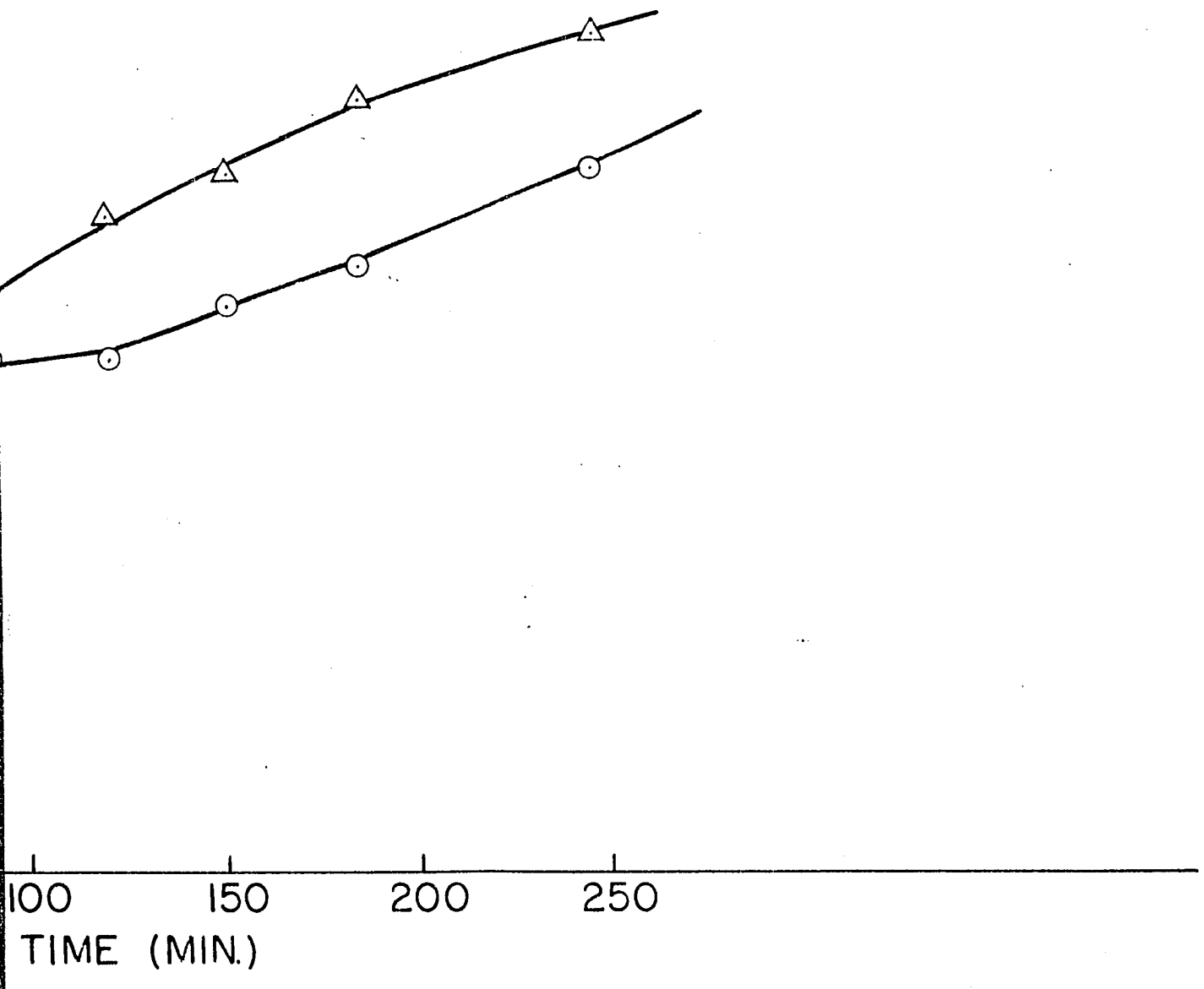
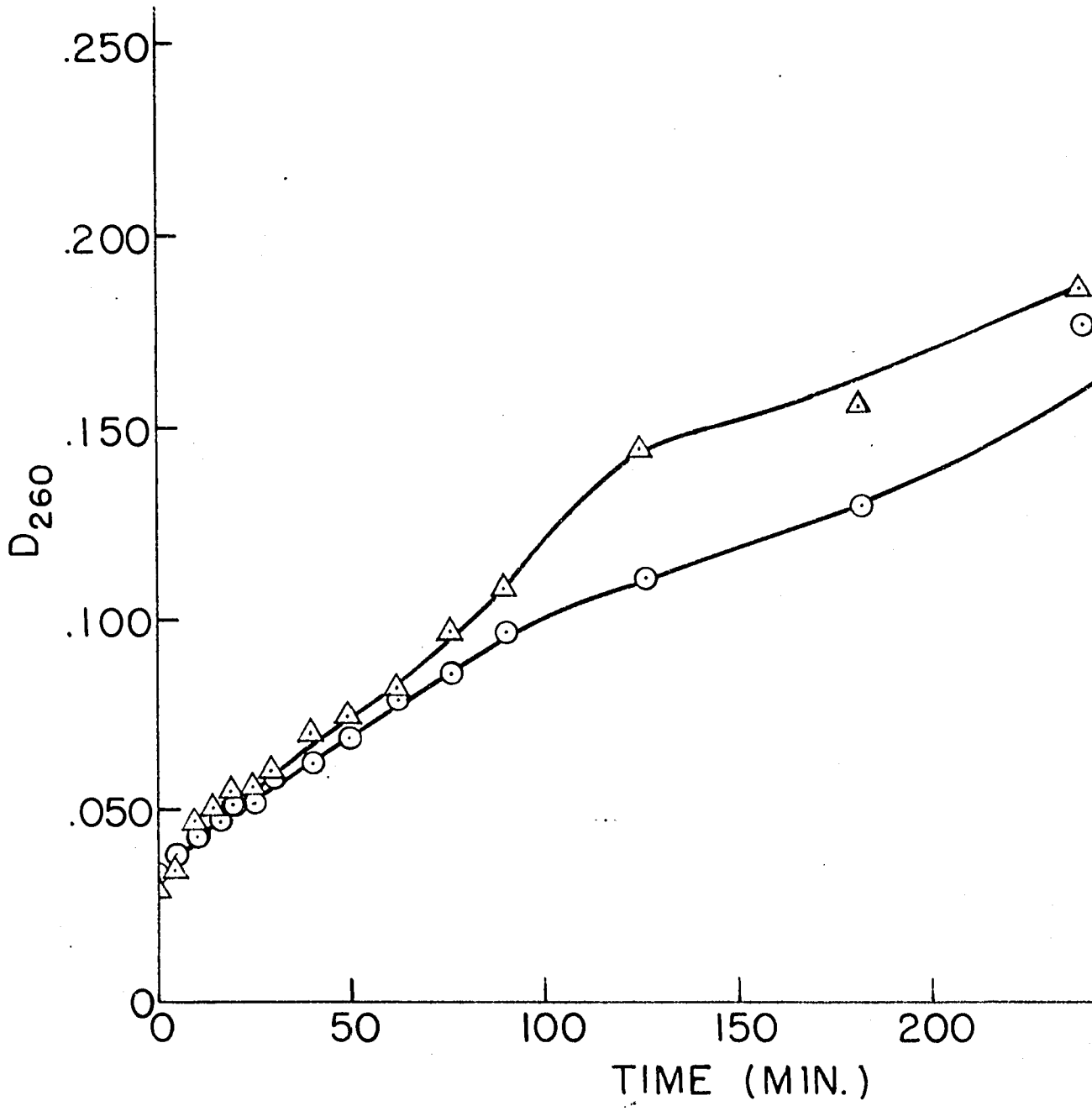

100
150
200
250
TIME (MIN.)

Figure 6. D_{260} of a 1/6 dilution of the trichloroacetic acid soluble fraction at various times after infection

⊙ Infection with unirradiated phage.

Δ Infection with irradiated phage.

Conditions of infection as in Figure 4.


.250
.200
.150
.100
.050
0
D_{260}
0
50
100
150
200
TIME (MIN.)

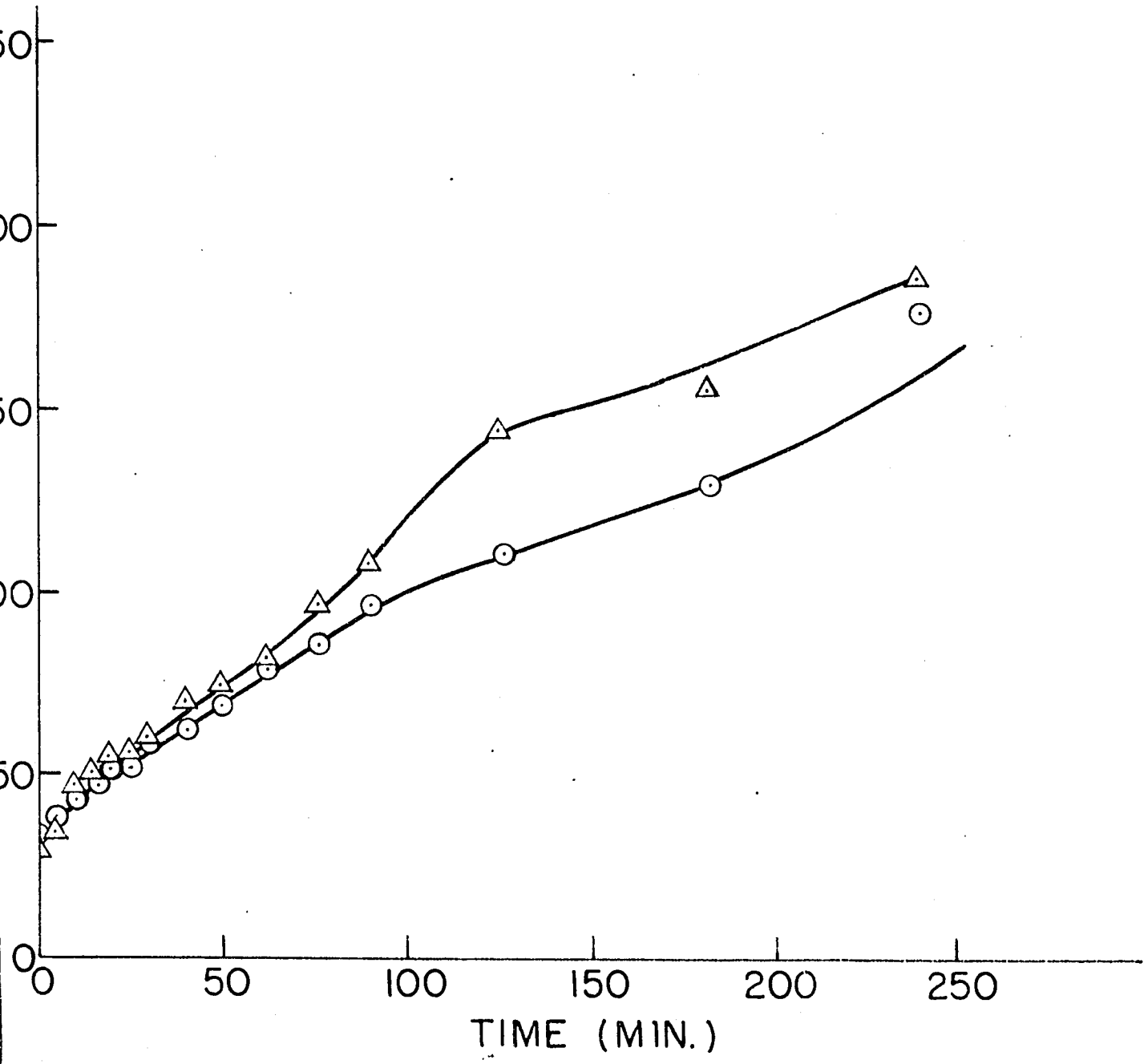

0
50
100
150
200
250
TIME (MIN.)

Table 7. UV absorption data from Experiment A

I. Optical densities (Six-fold dilution) - Infection with unirradiated phage

Wavelength in mμ	250	260	270	280	290	300
1) Sample drawn at 0 min.	.051	.061	.048	.026	.010	.003
2) Sample drawn at 35 "	.068	.075	.060	.034	.015	.005
3) Sample drawn at 90 "	.119	.135	.114	.073	.038	.018
4) Sample drawn at 245 "	.167	.184	.163	.115	.068	.035
2) minus 1) (above)	.017	.014	.012	.008	.005	.002
3) " 2) "	.051	.060	.054	.039	.023	.013
4) " 3) "	.048	.049	.049	.042	.030	.017

II. Optical densities (Six-fold dilution) - Infection with irradiated phage

Wavelength in mμ	250	260	270	280	290	300
5) Sample drawn at 0 min.	.052	.058	.051	.031	.015	.010
6) Sample drawn at 35 "	.079	.081	.068	.042	.019	.010
7) Sample drawn at 90 "	.137	.152	.132	.085	.042	.018
8) Sample drawn at 245 "	.179	.216	.195	.135	.073	.035
6) minus 5) (above)	.027	.023	.017	.011	.004	.000
7) " 6) "	.058	.071	.064	.043	.023	.009
8) " 7) "	.042	.064	.063	.040	.031	.017

Examination of the spectra reveals peaks at 260 mμ which are characteristic of nucleic acids and its breakdown products down to the purine and pyrimidine bases. The presence also of other UV absorbing substances such as protein derivatives is indicated, however, particularly in the optical density differences between samples drawn at two different

times after infection.

There appears to be no indication of a build-up of UV absorbing acid soluble materials in the case of infection with irradiated phage such as might be expected should a block in the nucleic acid synthesis be imposed by the radiation. In fact, as was mentioned previously, there appears to be a negligible difference between the cases of infection with irradiated phage and with unirradiated phage for the first 60 minutes as far as UV absorbing acid soluble materials are concerned.

One-Step Growth Experiment

In order to test for differences in length of the latent period and burst size between infections with UV irradiated and unirradiated T_2r+ bacteriophage pairs of one-step growth experiments (47) were performed with multiple infection of the bacterial cells by the phage.

The procedure (modified for use in this experiment) consists essentially of the following steps. E. coli B. cells grown to a concentration of approximately 1×10^9 per ml. in nutrient supplemented M-9 medium are washed and resuspended in phage buffer. The T_2r+ (irradiated or unirradiated) are added to a final concentration of $3\text{-}4 \times 10^9$ phage per ml. Several minutes at $37^\circ C$ are allowed for phage adsorption. At zero time the appropriate proportion of a concentrated supplemented M-9 medium is added. At 10 minutes an aliquot of the mixture is diluted in the growth medium to a final concentration of approximately 1×10^3 cells per ml. and a second aliquot of the mixture is diluted in a similar manner to a concentration of about 20 cells per ml. Platings are made at intervals beginning at 18 minutes for plaque counts.

At first the number of plaques from tubes at the higher concentration will be suitable for counting with few or no plaques on the plates from the lower concentration of cells. Later, however, after the cells have burst the numbers of plaques on the former plates will be too numerous to count while the numbers on the latter plates will be suitable for counting.

The data from one of these experiments are plotted in Figure 7. There appears to be very little difference in the time of initial appearance of new virus in the UV irradiated and unirradiated cases. The rate of increase of new virus may be greater in the case of infection with unirradiated phage, however. The burst size after infection with unirradiated T_2r+ was about 100 compared with 50 for the case of infection with the UV irradiated phage, which compares to data collected from phage yields in previous experiments.

Premature Lysis

It is of interest to investigate the development of active phage particles in cells before the end of the latent period in the case of multiple infection with UV irradiated phage and to compare this with phage development after infection with unirradiated phage. In order to accomplish this it is necessary to cause the cells to lyse prematurely without inactivating the intracellular virus.

The lysing medium used for this purpose in these experiments contained cyanide, as a metabolic inhibitor to stop the progress of phage development at a precise moment, and chloroform, to prematurely lyse the infected cells. The concentrations of these substances in the lysing

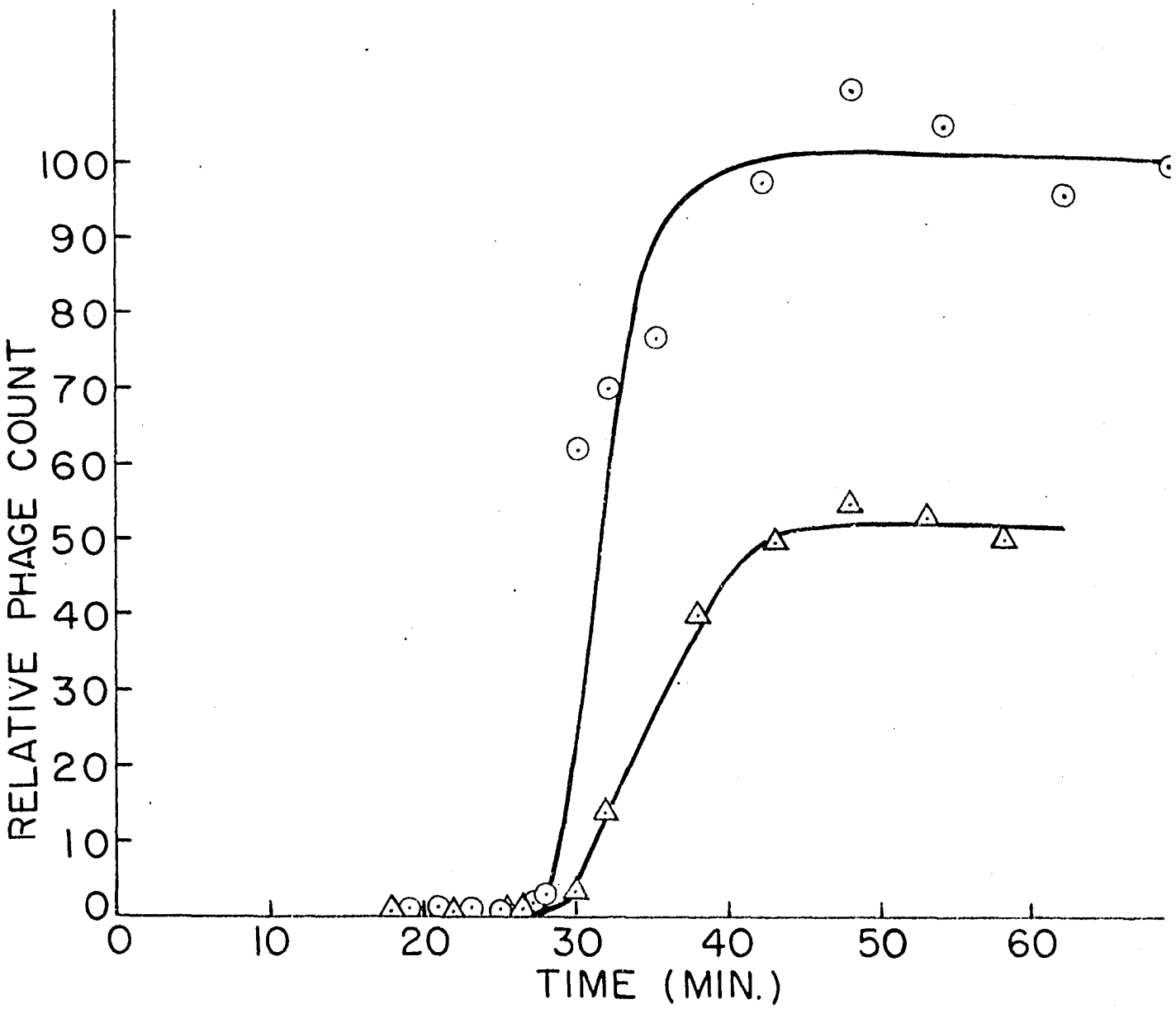
Figure 7. One-step growth experiment. Relative phage titer at various times after infection

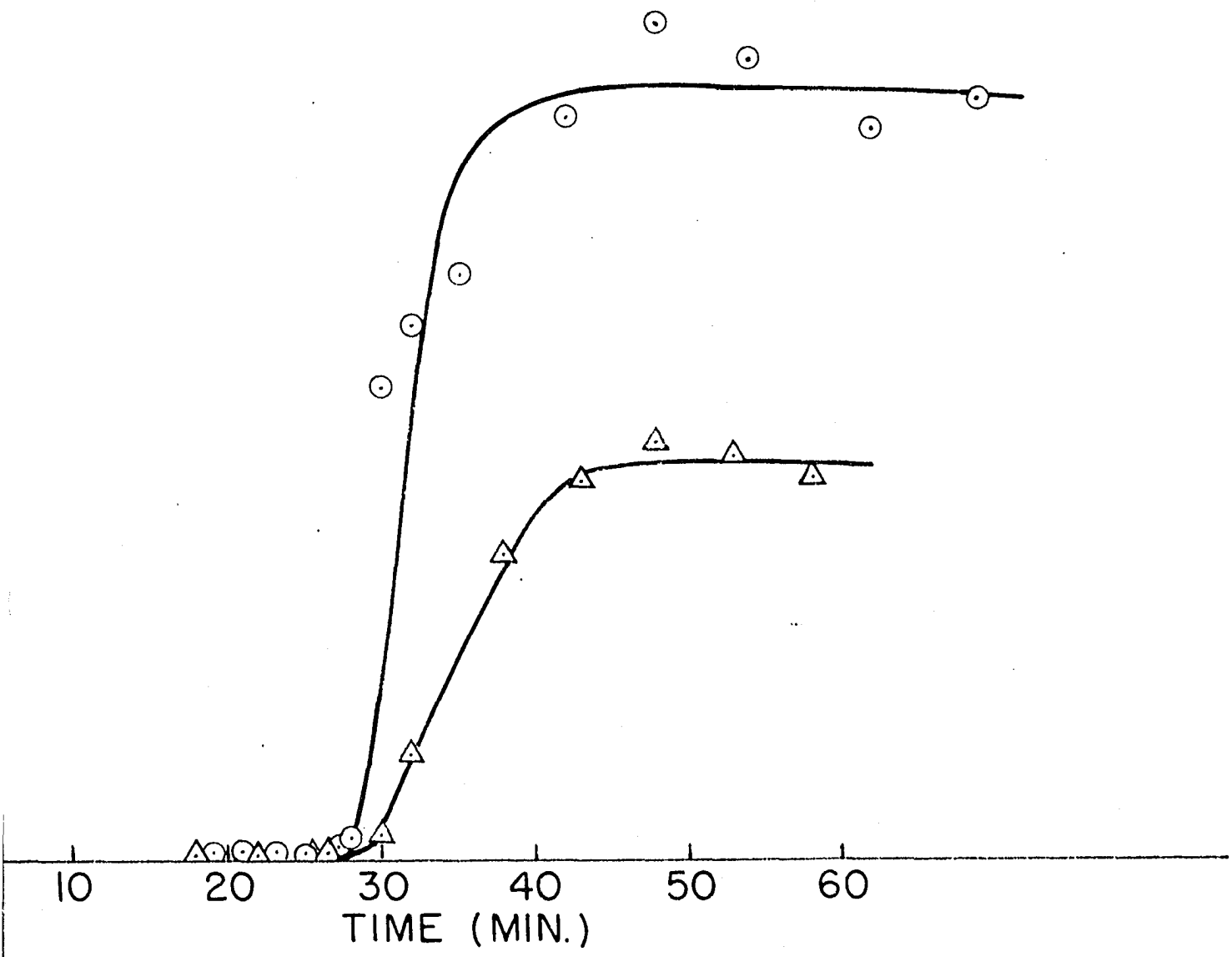
O Infection with unirradiated phage.

Δ Infection with irradiated phage (survival = 0.006).

Original concentration of bacteria = 8×10^8/ml.

Actual multiplicity of infection = 4.0.


RELATIVE PHAGE COUNT
0
10
20
30
40
50
60
70
80
90
100
0
10
20
30
40
50
60
TIME (MIN.)


10
20
30
40
50
60
TIME (MIN.)

medium have been given in the materials section of this paper.

The E. coli B. cells were grown in M-9 medium to a concentration of approximately 7 x 10^8 cells per ml. After removing an aliquot for assay of the bacterial concentration the suspension was divided into two parts, one of which was infected with UV irradiated phage and the other with unirradiated phage at identical multiplicities of about 4 at zero time.

One ml. aliquots from these cultures were transferred at desired times (usually 2 minute intervals) to 9 ml. of the lysing medium emulsion and mixed thoroughly. After the tubes had stood for 10 or 15 minutes, aliquots from each of these tubes, after serial dilution, were plated out for plaques on agar plates.

The results of this experiment are graphed and appear in Figure 8.

The delay in appearance of intracellular active phage particles in the case of multiple infection with UV irradiated T_2r+ appears to parallel the delay in increase in total DNA as indicated in previous experiments, with these irradiated phage. In this experiment, as in former experiments, the delay appears to be approximately 8 minutes in comparison with the process in infection with unirradiated phage.

Determination of Increase of Bacteriophage DNA with Time

Since the curve for total DNA versus time after infection of E. coli B. in the case of infection with UV irradiated T_2r+ is similar to that for infection with unirradiated T_2r+ except for corresponding points falling 8 - 10 minutes later, it may be reasonable to assume that the synthesis of T_2r+ DNA in this case is delayed 8 - 10 minutes behind the synthesis

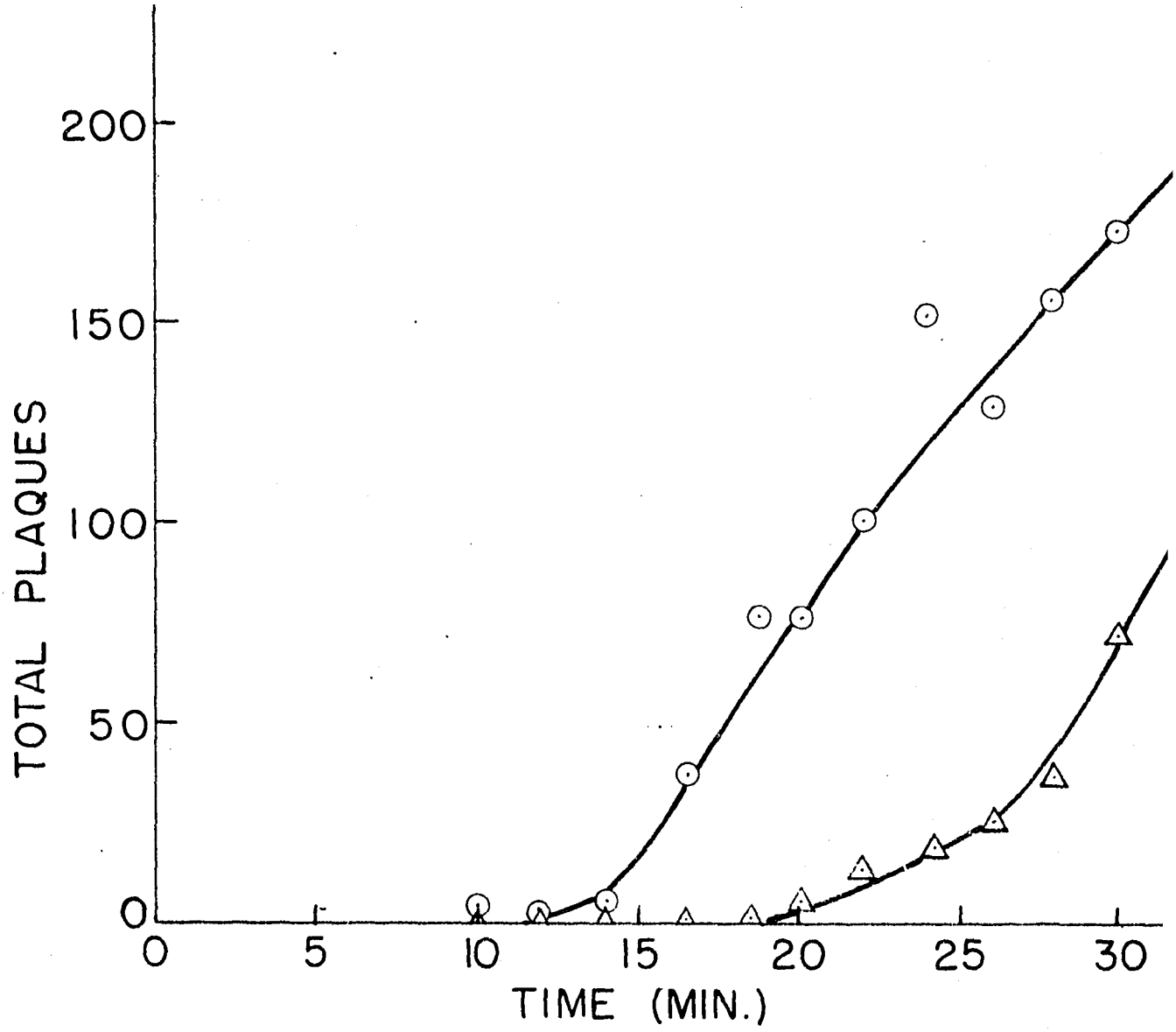
Figure 8. Intracellular phage development at various times after infection

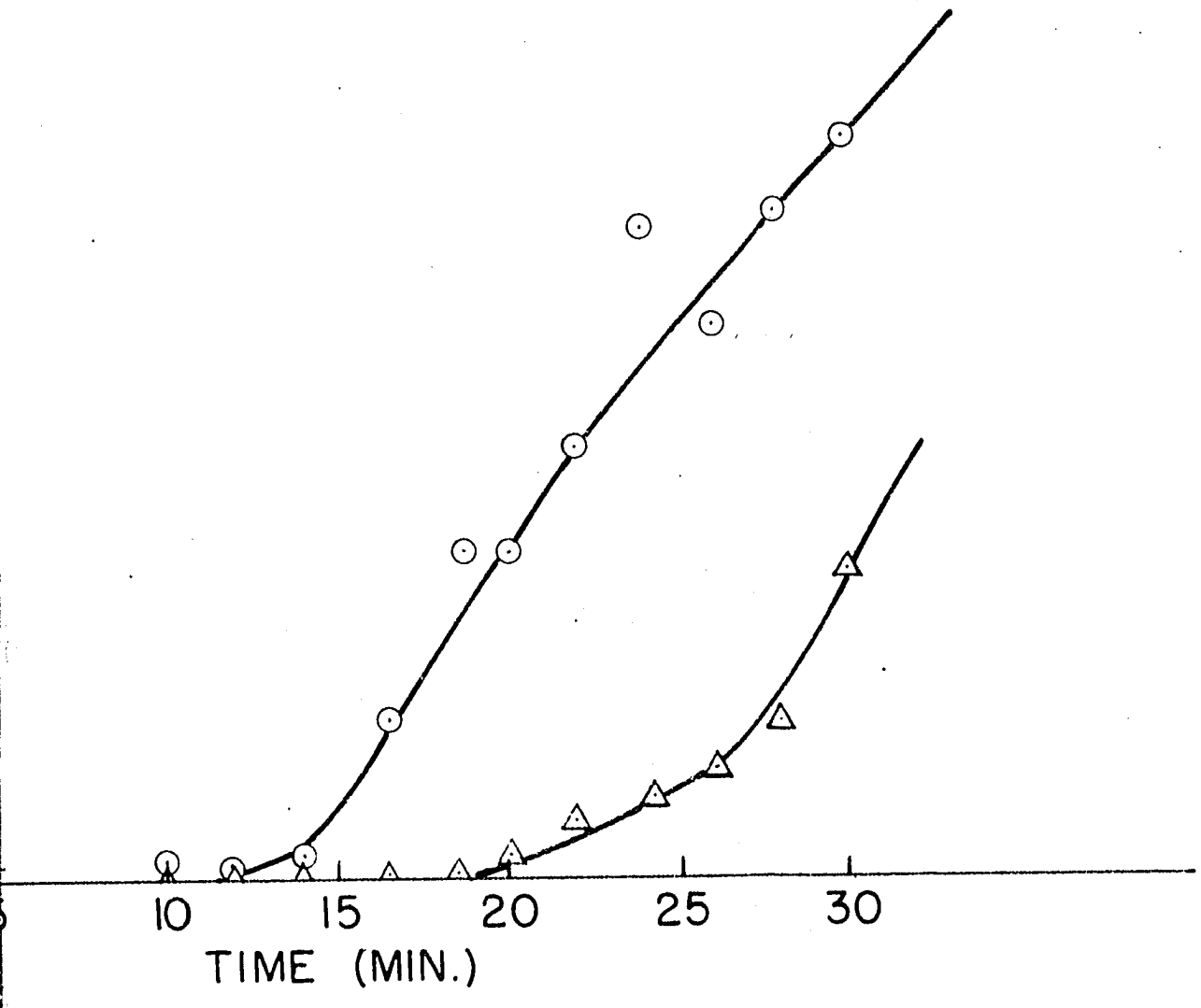
⊙ Infection with unirradiated phage.

Δ Infection with irradiated phage (survival = 0.004).

Original concentration of bacteria = 7×10^8/ml.

Actual multiplicity of infection = 3.0.


TOTAL PLAQUES
200
150
100
50
0
0
5
10
15
20
25
30
TIME (MIN.)


10
15
20
25
30
TIME (MIN.)

of T_2r+ DNA after infection with unirradiated phage. This assumes, of course, that any changes in the amount of bacterial DNA which take place with time are the same in the two cases since the total DNA is made up of E. coli B. DNA plus phage T_2r+ DNA. Such an assumption is not actually justified, however, until it has been experimentally verified.

Experiments were therefore designed and performed to measure the fraction of the total DNA which was T_2r+ DNA at various times after phage infection. The general method employed in these experiments has been outlined in some detail in a preceding section and will not be repeated here.

Figure 9 gives the results of the first experiment of this type. The bacterial DNA and the phage DNA are plotted separately against the time after infection. The curves are self explanatory. It can be seen that there is actually a lag in the synthesis of phage DNA in bacterial cells with a multiple infection of UV irradiated T_2r+. In this experiment the readings were taken at intervals of approximately 5 minutes up to 35 minutes after infection. Because of this the detail in the first parts which are the significant portions of the curves was not clear without further investigation. A second experiment of a similar nature was undertaken with samples drawn more often but over a shorter time period. The results are plotted in Figure 10. Although the processes were initiated a little slower than in the previous experiment, perhaps due to lower aeration, the results are similar with the lag again obvious. This experiment seems to indicate that there is no viral DNA synthesis in the first few minutes after infection in the case of multiple infection with the UV

Figure 9. Bacterial DNA, phage DNA, and intracellular phage titer at various times after infection

▲ Bacterial DNA in bacteria infected with unirradiated phage.

Δ Bacterial DNA in bacteria infected with irradiated phage.

● Phage DNA in bacteria infected with unirradiated phage.

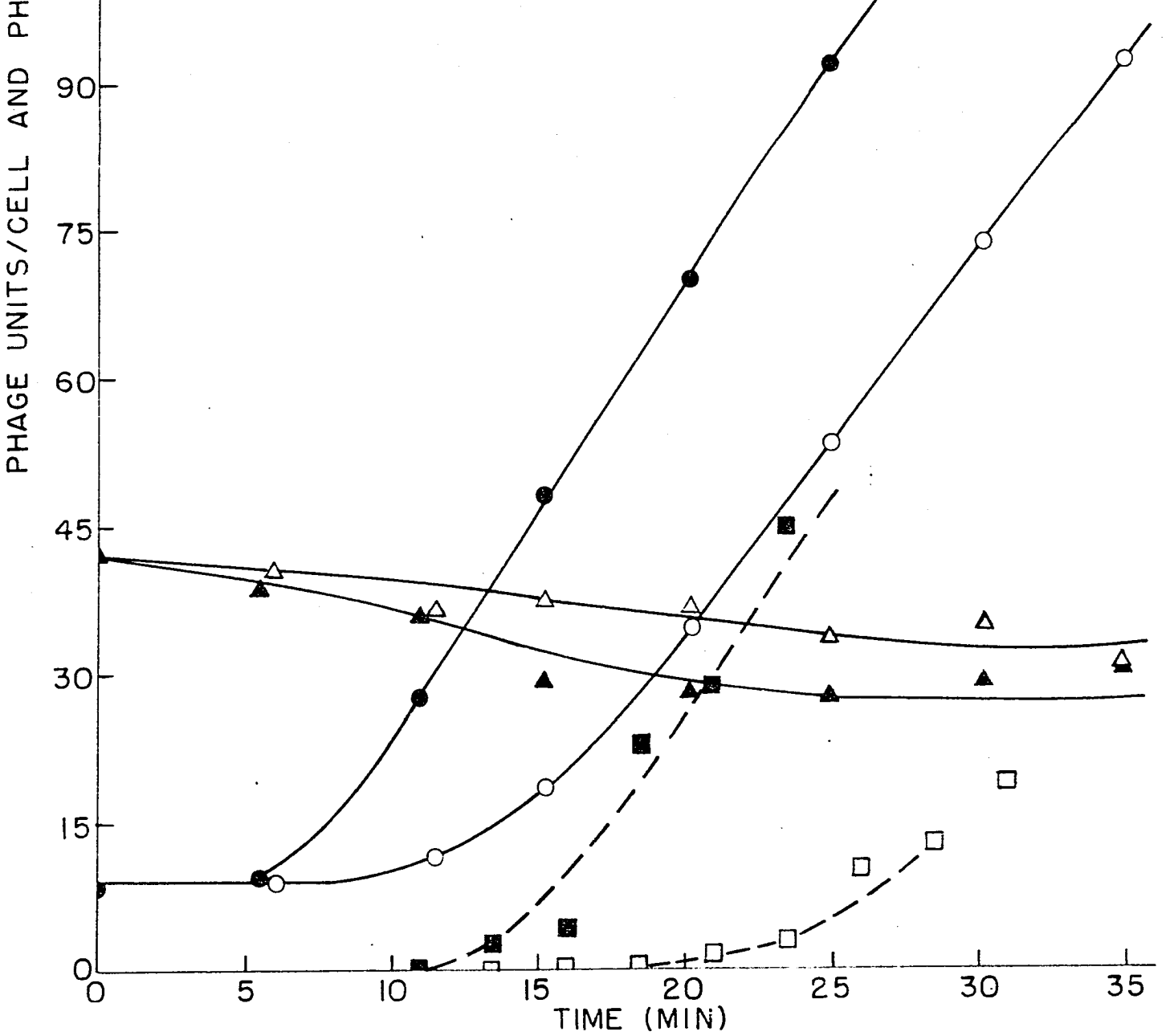
O Phage DNA in bacteria infected with irradiated phage.

■ Intracellular phage/cell in bacteria infected with unirradiated phage.

□ Intracellular phage/cell in bacteria infected with irradiated phage.

Original concentration of bacteria = 1×10^9/ml.

Actual multiplicity of infection = 7.0.


PHAGE UNITS/CELL AND PH
90
75
60
45
30
15
0
0
5
10
15
20
25
30
35
TIME (MIN)

PHAGE UNITS/CELL AND PHAGE/CELL

60 75 90 105 120 135 150 165

Figure 10. Bacterial DNA and phage DNA at various times after infection

▲ Bacterial DNA in bacteria infected with unirradiated phage.

Δ Bacterial DNA in bacteria infected with irradiated phage.

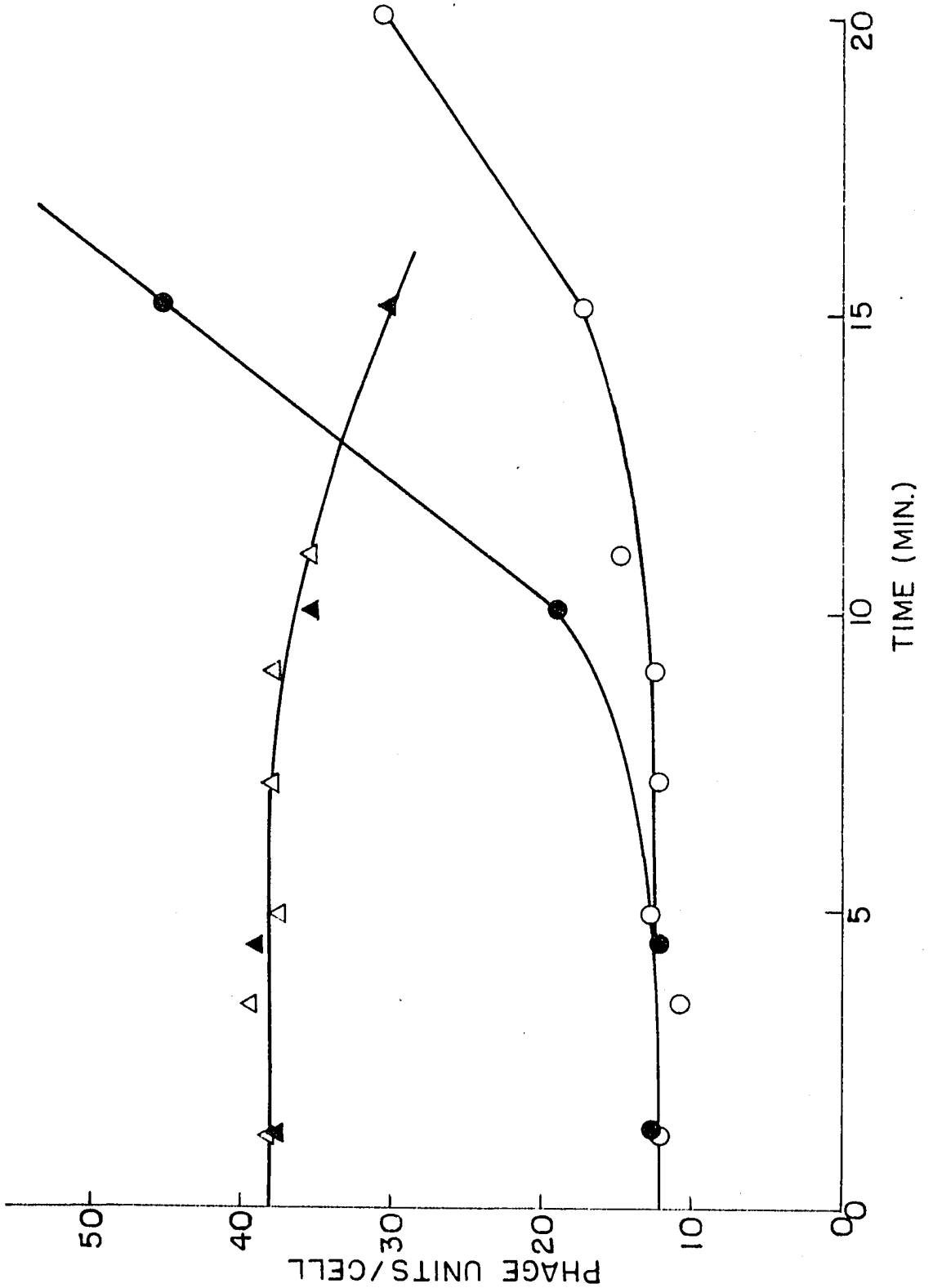
● Phage DNA in bacteria infected with unirradiated phage.

O Phage DNA in bacteria infected with irradiated phage.

Original concentration of bacteria = 9×10^8/ml.

Actual multiplicity of infection = 6.0.

Survival of irradiated phage = 0.0067.


PHAGE UNITS/CELL
50
40
30
20
10
0
0
5
10
15
20
TIME (MIN.)

irradiated phage since the curve has zero slope for the first several minutes.

At this point it might be well to mention the 0.62 which is divided into the number of moles of DNA-P x 10^7 (see step 23 in the procedure under paper electrophoresis in a preceding section). The recovery of bacterial DNA is less than that of viral DNA in the separation of the DNA from the RNA plus protein prior to acid hydrolysis and paper electrophoresis of the free bases. Hershey and co-workers (2) also made note of this in their experiments. This effect may have something to do with the difference in the strength or space relationship of the bonding of DNA to protein in the bacteria compared with the virus. Since the number of moles of DNA-P from the bacteria is based on the total moles of DNA-P and on the fraction of the total DNA-P which is of bacterial origin, any difference in recovery of DNA of the different kinds must be corrected in such a way as to give the actual fraction of the total DNA-P originally present which was of bacterial origin. That this correction is approximately constant for all samples drawn was indicated by the fact that the points plotted without correction lay nearly on smooth curves. However, the point at zero time for T_2r+ DNA, which was determined on the basis of the numbers of phage added, was found to lie lower than the end point of the extrapolated value at zero time from the uncorrected curve for the data in the second experiment. In order to determine the value of the approximate correction necessary due to the smaller recovery of bacterial DNA than viral DNA, the coincidence of the extrapolated point at zero time with the experimentally determined one was chosen as the

criterion. It was found that dividing the bacterial DNA-P values by 0.62 made these two points coincide, and the data plotted in Figure 10 has been corrected accordingly. Applying the same value of 0.62 in the case of the data in the first experiment made the extrapolated value from the curves coincide with the experimentally determined zero point value. The graphs in Figure 9 are plotted also then from the data thus corrected.

The advantage of the method used in these experiments over those of Hershey (2) is that, whereas each of his experimental points for phage DNA versus time after infection was from a different experimental culture, the points plotted for each curve in Figures 9 and 10 are from a single culture. Also, the curves in a single figure for infection with UV irradiated and unirradiated T_2r+ are derived from the same bacterial culture and the same phage stocks, the only difference being that the phage in one case were irradiated with ultraviolet light to a survival less than 0.01.

In Figure 9 curves for intracellular phage in each of the two cases have also been plotted from data on samples drawn from the same cultures used for the determination of phage DNA. The method involved was identical to that described in the preceding section except that here the volume measurements were subject to some difficulty, being pipetted from an actively aerated culture which resulted, at times, in some bubbles in the pipettes. The curves resemble, however, the ones from the preceding section and enable an estimation of the phage DNA pool sizes to be made for infection with irradiated phage and with unirradiated phage. As may be seen in Figure 9 the pool size is about the same

in the two cases with about 45-50 phage units per cell of phage DNA in excess of the active intracellular phage at any time after the intracellular phage begin to appear.

Experiments Using 5-Methyl Tryptophane

Since 5-methyl tryptophane has been observed to inhibit protein synthesis without affecting DNA synthesis it has been used in studies relating the synthesis of phage DNA to the synthesis of protein in phage reproduction (36). Chloramphenicol has been used for a similar purpose (35). Since 5-methyl tryptophane was available in our laboratory it was chosen for use in multiplicity reactivation experiments. Three experiments using it were performed. In the first experiment UV irradiated T_2r+ were used in a multiple infection of E. coli B. in M-9 medium containing 5×10^{-4} M. 5-methyl tryptophane. At 13 minutes dl-tryptophane was added to a final concentration of 1×10^{-3} M. to reverse the inhibition of the 5-methyl tryptophane. Two controls were used which were identical to the test culture except that the dl-tryptophane was not added at 13 minutes. In one of the controls the dl-tryptophane (to a final concentration of 1×10^{-3} M.) was added simultaneously with the 5-methyl tryptophane at zero time. No dl-tryptophane at all was added in the other control. Viral synthesis was followed by checking for the development of active intracellular phage by premature lysis using the techniques employed previously.

The second experiment using 5-methyl tryptophane was similar to the first except that multiple infection with unirradiated T_2r+ took the

place of multiple infection with irradiated phage.

The results of these two experiments are shown in Figures 11 and 12. In each experiment the delay in the development of intracellular phage, in the case where the 5-methyl tryptophane was not reversed for 13 minutes after infection, was 12 minutes or thereabouts. This indicates that some kind of protein synthesis early in the process is required in the case of multiplicity reactivation as well as in multiple infection with unirradiated T_2r+ bacteriophage.

It is not clear what this process can be, but it possibly involves the synthesis of enzymes necessary for the initial steps in multiplicity reactivation.

The third experiment with 5-methyl tryptophane (using M-9 again as the culture medium) utilized both UV irradiated and unirradiated T_2r+ with the further modification that in each of these cases the 5-methyl tryptophane was added at 7 minutes after addition of the phage rather than with the phage at zero time. Actually the bacteria, after reaching the desired concentration, were divided into four culture flasks with UV irradiated T_2r+ added to two of the flasks and unirradiated T_2r+ added to the other two. The 5-methyl tryptophane was added at 7 minutes to each, as indicated above, but in one irradiated and one unirradiated flask the inhibition was reversed by the addition of tryptophane at 22 minutes, whereas no tryptophane was added at any time to the other pair. The synthetic processes were followed by assaying for total DNA in the flasks where no tryptophane was added for reversal of the inhibition. In the other pair the development of active intracellular phage was followed with assays of the total DNA taken at two times for comparison with the

Figure 11. Effect of 5-methyltryptophane (5 MT) and dl-tryptophane (T) upon the development of intracellular phage (irradiated)

● Phage/cell at various times after infection. 5 MT (5×10^{-4} M) and T (10^{-3} M) added at zero time.

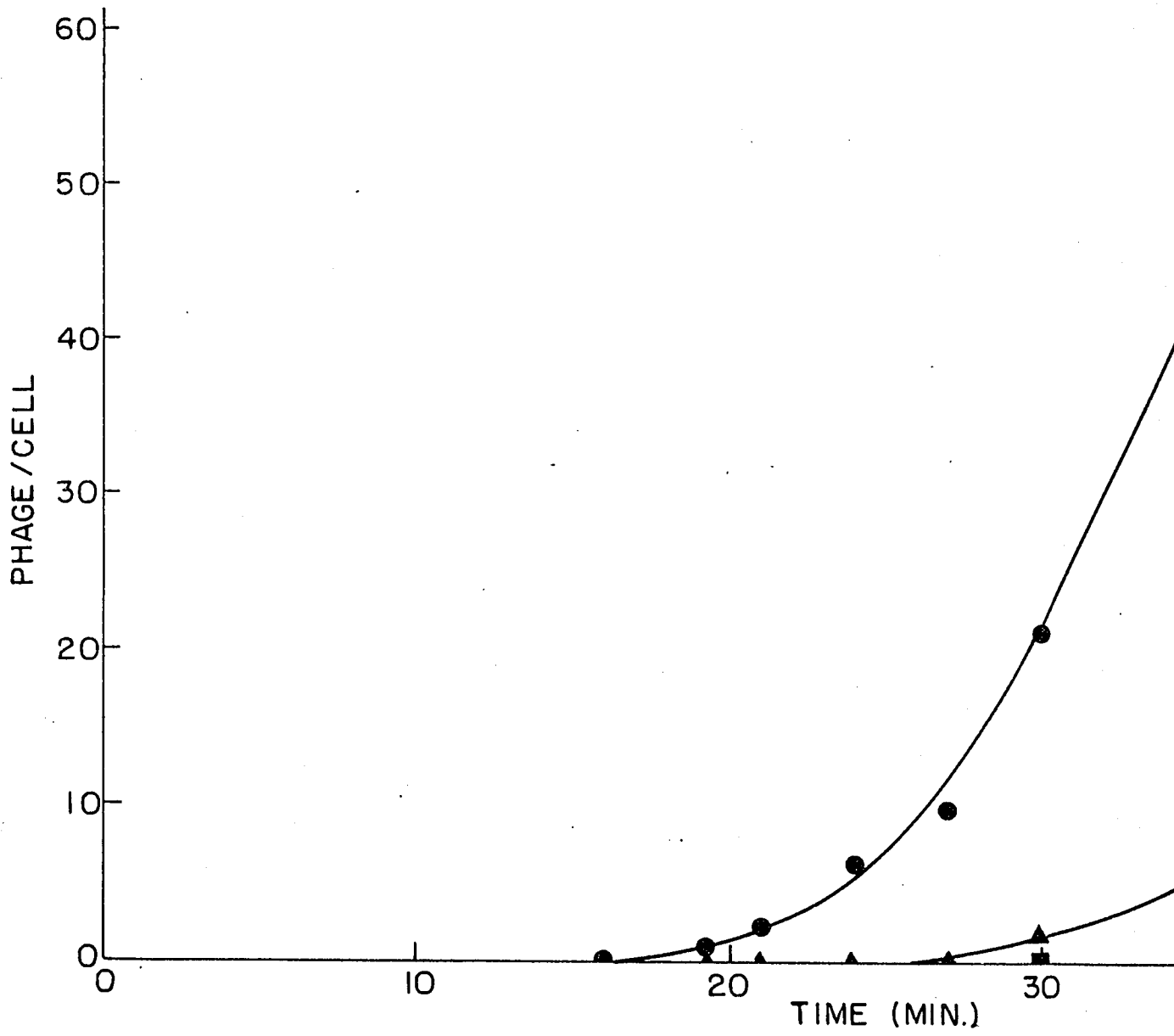
▲ Phage/cell at various times after infection. 5 MT (5×10^{-4} M) added at zero time, T(10^{-3} M) added at 13 minutes.

■ Phage/cell at various times after infection. 5 MT (5×10^{-4} M) added at zero time.

Original concentration of bacteria = 9.6×10^{8}/ml.

Actual multiplicity of infection = 7.0.

All phage had been irradiated to a survival of 0.006.


60
50
40
30
20
10
0
PHAGE/CELL
0
10
20
30
TIME (MIN.)

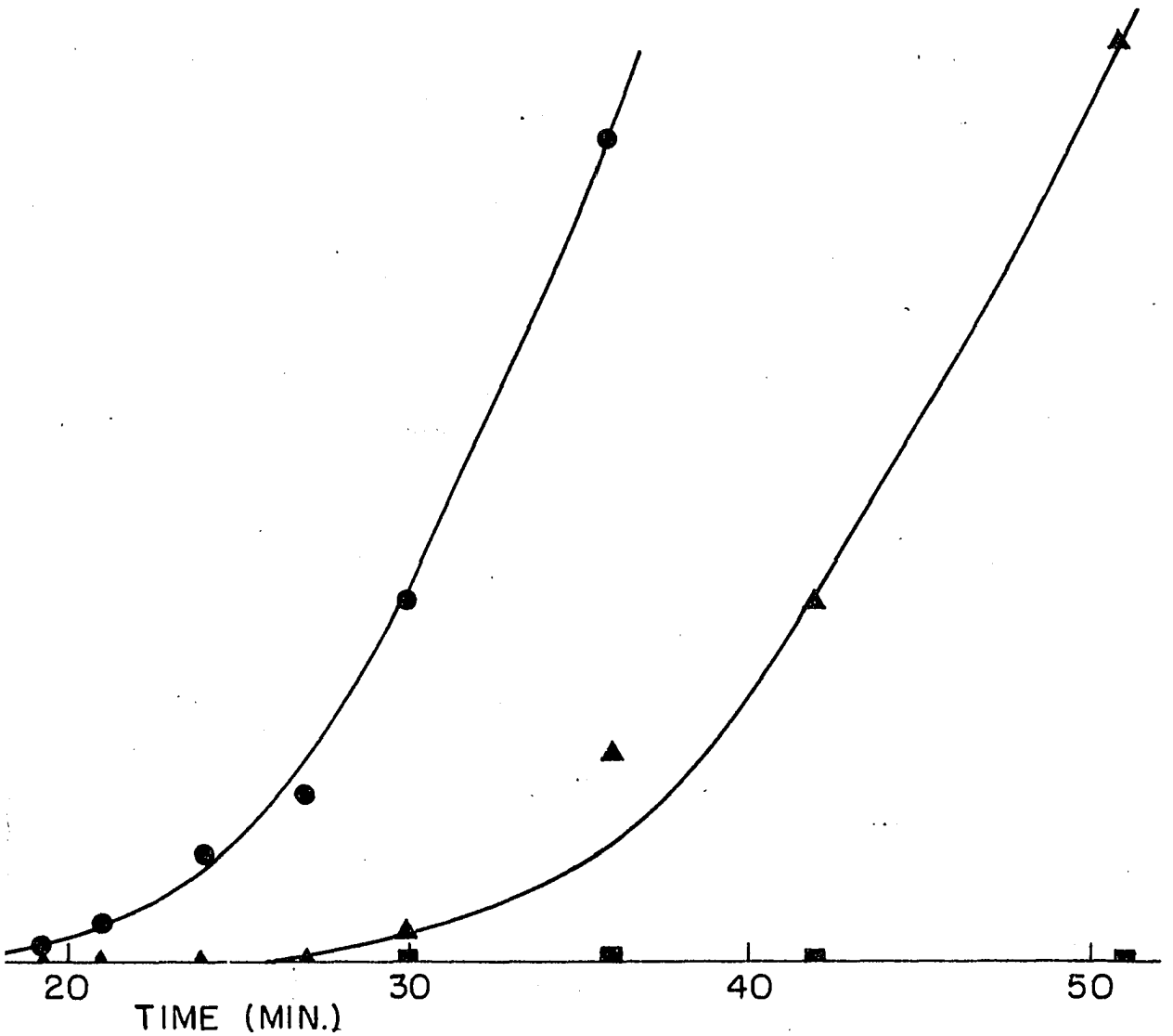

20
30
40
50
TIME (MIN.)

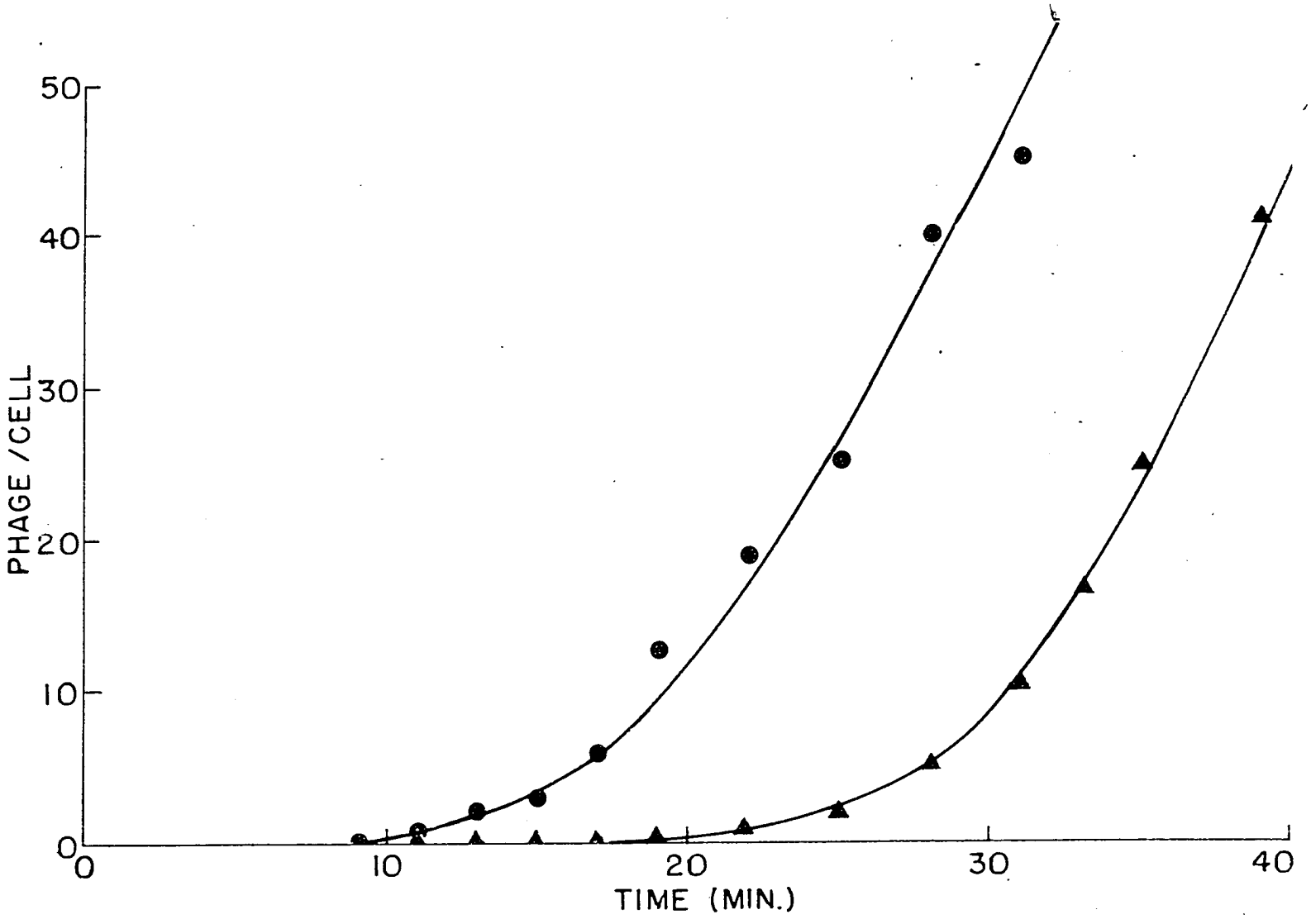
Figure 12. Effect of 5-methyltryptophane (5 MT) and dl -tryptophane (T) upon the development of intracellular phage (unirradiated)

● Phage/cell at various times after infection. 5 MT (5×10^{-4} M) and T (10^{-3} M) added at zero time.

▲ Phage/cell at various times after infection. 5 MT (5×10^{-4} M) added at zero time and T(10^{-3} M) added at 13 minutes.

Original concentration of bacteria = 9×10^{8}/ml.

Actual multiplicity of infection = 7.0.


50
40
30
20
10
0
PHAGE /CELL
0
10
20
30
40
TIME (MIN.)

total DNA at corresponding times in the cultures with no tryptophane added.

A schedule of operations for this experiment, which is typical of the schedules used for many of the experiments, is given in Table 8. A separate column is used for each of the four flasks A, B, C, and D. It will be noticed that since the same operation cannot be performed on each of the four flasks simultaneously, zero times (and consequently the actual times for the succeeding operations) are spaced one minute apart for each of the flasks. The tubes containing cold trichloracetic acid used for the samples for total DNA assay by the Dische diphenylamine test are indicated with unprimed numbers with the letters accompanying the numbers representing the flask from which the sample was withdrawn; the tubes used to hold the lysing solution in the assay for active intracellular phage particles are indicated with primed numbers with the letters having the same significance as before. The times listed are the times when the samples actually contacted the TCA or cyanide solution thus stopping all metabolic activity.

The results of this experiment are found in Figures 13 and 14, the total DNA versus time being plotted in Figure 13 and active intracellular phage being plotted in Figure 14. In this case the total DNA has been expressed in terms of phage units per cell as in certain previous experiments.

According to the curve for infection with unirradiated T_2r+ in Figure 13, the effect of the addition of the 5-methyl tryptophane at 7 minutes is to prevent any increase in the rate of DNA synthesis beyond that which it had at that time, and to keep the rate constant at that value. In other words it does not stop DNA synthesis once under way, but prevents new

Table 8. Operation schedule for experiment using 5-methyl tryptophane

A	B	C	D
0 min. - add unirradiated phage	1 min. - add irradiated phage	2 min. - add unirradiated phage	3 min. - add irradiated phage
1/2 min. - 5 mls. from A into 1A	1 1/2 min. - 5 mls. from B into 1B		
4 min. - 0.1 ml. from A for phage adsorption check			
5 min. - 5 mls. from A into 2A	6 min. - 5 mls. from B into 2B		
7 min. - add 5 MT	8 min. - add 5 MT	9 min. - add 5 MT	10 min. - add 5 MT
9 min. - 5 mls. from A into 3A	10 min. - 5 mls. from B into 3B		
13 min. - 5 mls. from A into 4A	14 min. - 5 mls. from B into 4B		
17 min. - 5 mls. from A into 5A	18 min. - 5 mls. from B into 5B		
21 min. - 5 mls. from A into 6A	22 min. - 5 mls. from B into 6B	24 min. - add tryptophane	25 min. - add tryptophane
25 min. - 5 mls. from A into 7A	26 min. - 5 mls. from B into 7B	26 min. - 1 ml. from C into 1'C	27 min. - 1 ml. from D into 1'D
		30 min. - 1 ml. from C into 2'C	31 min. - 1 ml. from D into 2'D
29 min. - 5 mls. from A into 8A	30 min. - 5 mls. from B into 8B	31 min. - 5 mls. from C into 1C	32 min. - 5 mls. from D into 1D
		33 min. - 1 ml. from C into 3'C	34 min. - 1 ml. from D into 3'D

Table 8 (continued)

A	B	C	D
		36 min. - 1 ml. from C into 4'C	37 min. - 1 ml. from D into 4'D
		37 min. - 5 ml. from C into 2C	38 min. - 5 ml. from D into 2D
		39 min. - 1 ml. from C into 5'C	40 min. - 1 ml. from D into 5'D
		42 min. - 1 ml. from C into 6'C	43 min. - 1 ml. from D into 6'D
		45 min. - 1 ml. from C into 7'C	46 min. - 1 ml. from D into 7'D
		50 min. - 1 ml. from C into 8'C	51 min. - 1 ml. from D into 8'D
		55 min. - 1 ml. from C into 9'C	56 min. - 1 ml. from D into 9'D
		60 min. - 1 ml. from C into 10'C	61 min. - 1 ml. from D into 10'D

DNA synthesis. This is in agreement with Burton's observations (36) using 5-methyl tryptophane and the observations of Tomizawa and Sunakawa (35) using chloramphenicol as an inhibitor of protein synthesis. Accordingly, when the inhibition is reversed at 22 minutes with tryptophane, the rate of DNA synthesis is increased.

In accordance with the observation made above it is not surprising that upon infection with UV irradiated T_2^{r+} the rate of DNA synthesis is zero (until the inhibition is reversed) when 5-methyl tryptophane is

Figure 13. Effects of 5-methyltryptophane (5 MT) and dl -tryptophane (T) upon the DNA/cell during phage infection

● DNA/cell at various times after infection with unirradiated phage. 5 MT(5×10^{-4} M) added at 7 minutes.

▲ DNA/cell at various times after infection with irradiated phage (survival = 0.0038). 5 MT(5×10^{-4} M) added at 7 minutes.

O DNA/cell at various times after infection with unirradiated phage. 5 MT(5×10^{-4} M) added at 7 minutes and T(10^{-3} M) added at 22 minutes.

Δ DNA/cell at various times after infection with irradiated phage (survival = 0.0038). 5 MT(5×10^{-4} M) added at 7 minutes and T (10^{-3}M) added at 22 minutes.

Original concentration of bacteria = 1×10^{9}/ml.

Actual multiplicity of infection = 6.

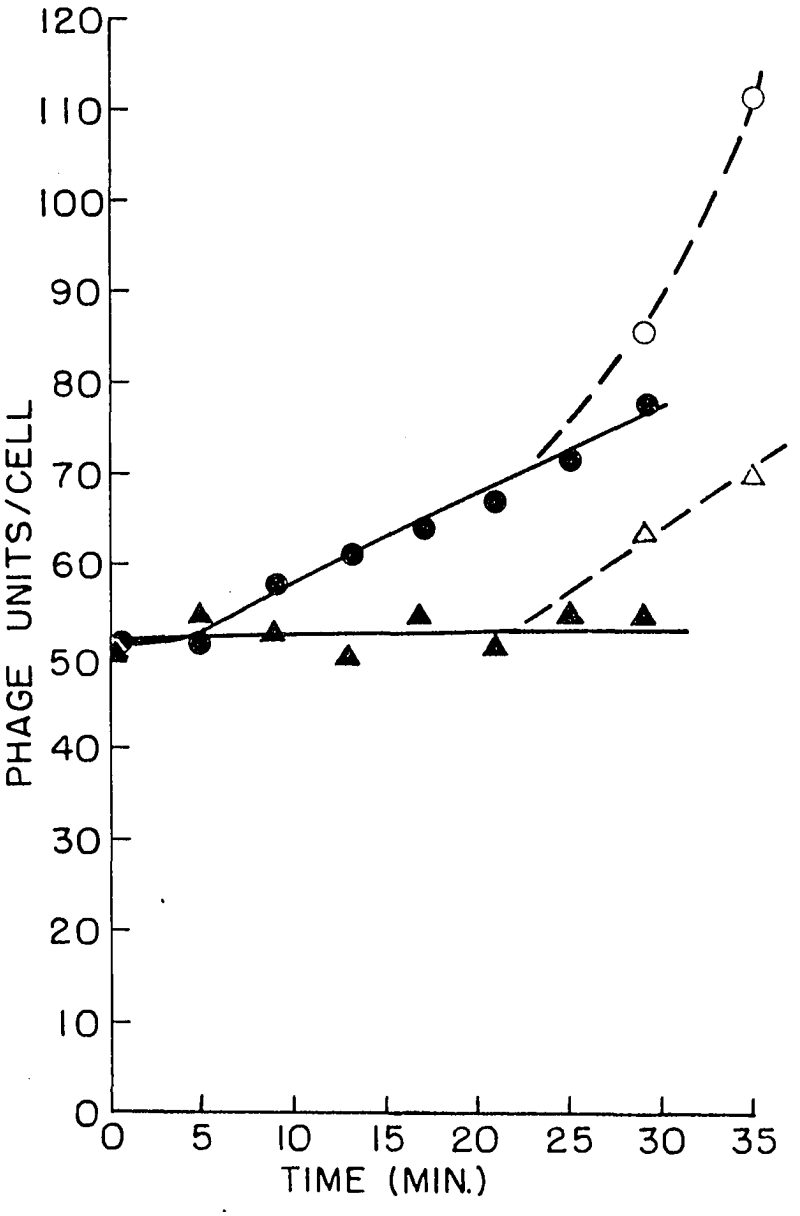

120
110
100
90
80
70
60
50
40
30
20
10
0
PHAGE UNITS/CELL
0
5
10
15
20
25
30
35
TIME (MIN.)

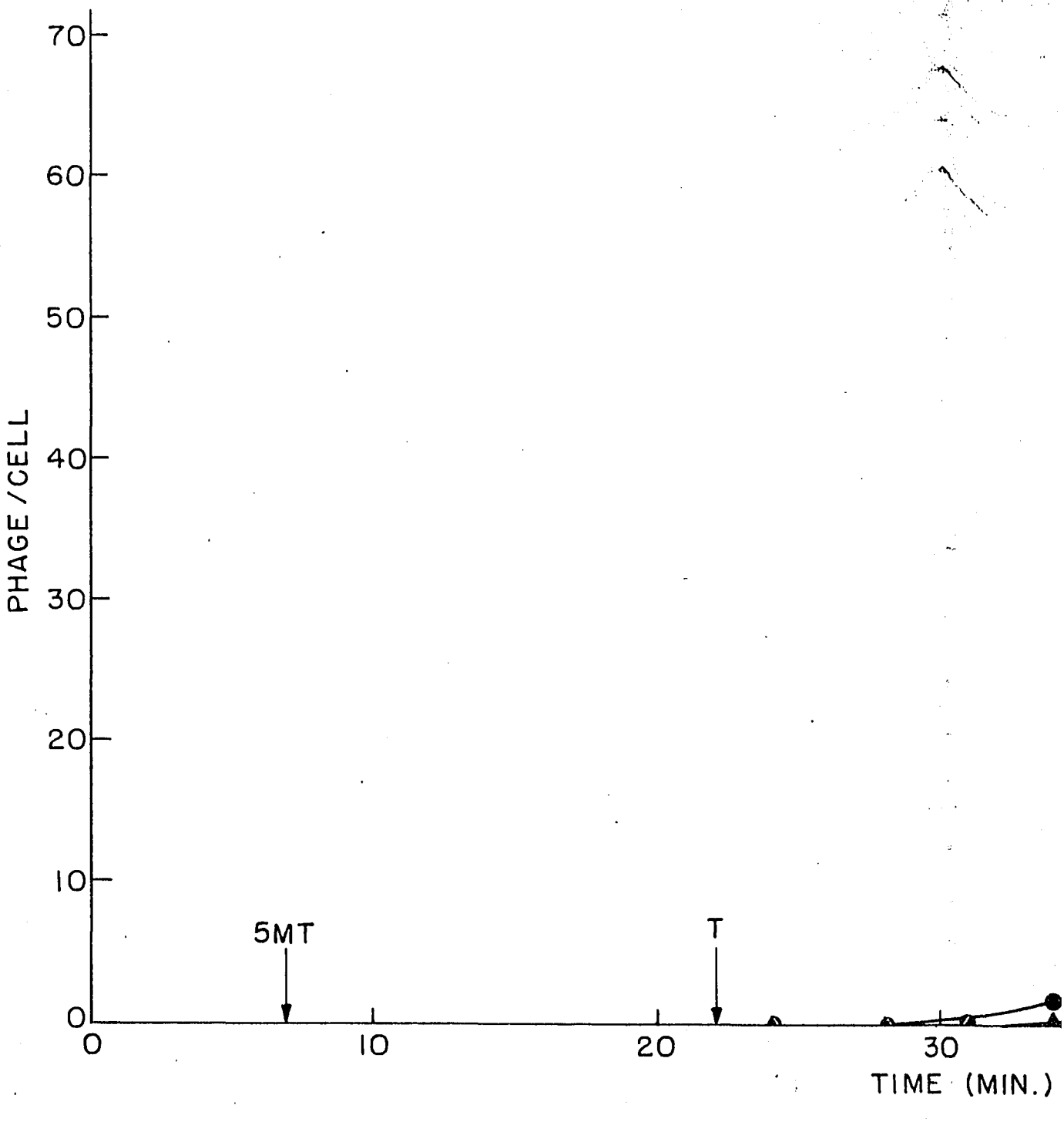
Figure 14. Effects of 5-methyltryptophane (5 MT) and dl -tryptophane (T) upon the development of intracellular phage

● Phage/cell at various times after infection with unirradiated phage. 5 MT(5×10^{-4} M) added at 7 minutes and T(10^{-3} M) added at 22 minutes.

▲ Phage/cell at various times after infected with irradiated phage (survival = 0.0038). 5 MT(5×10^{-4} M) added at 7 minutes and T(10^{-3} M) added at 22 minutes.

Original concentration of bacteria = 1×10^9/ml.

Multiplicity of infection = 6.


70
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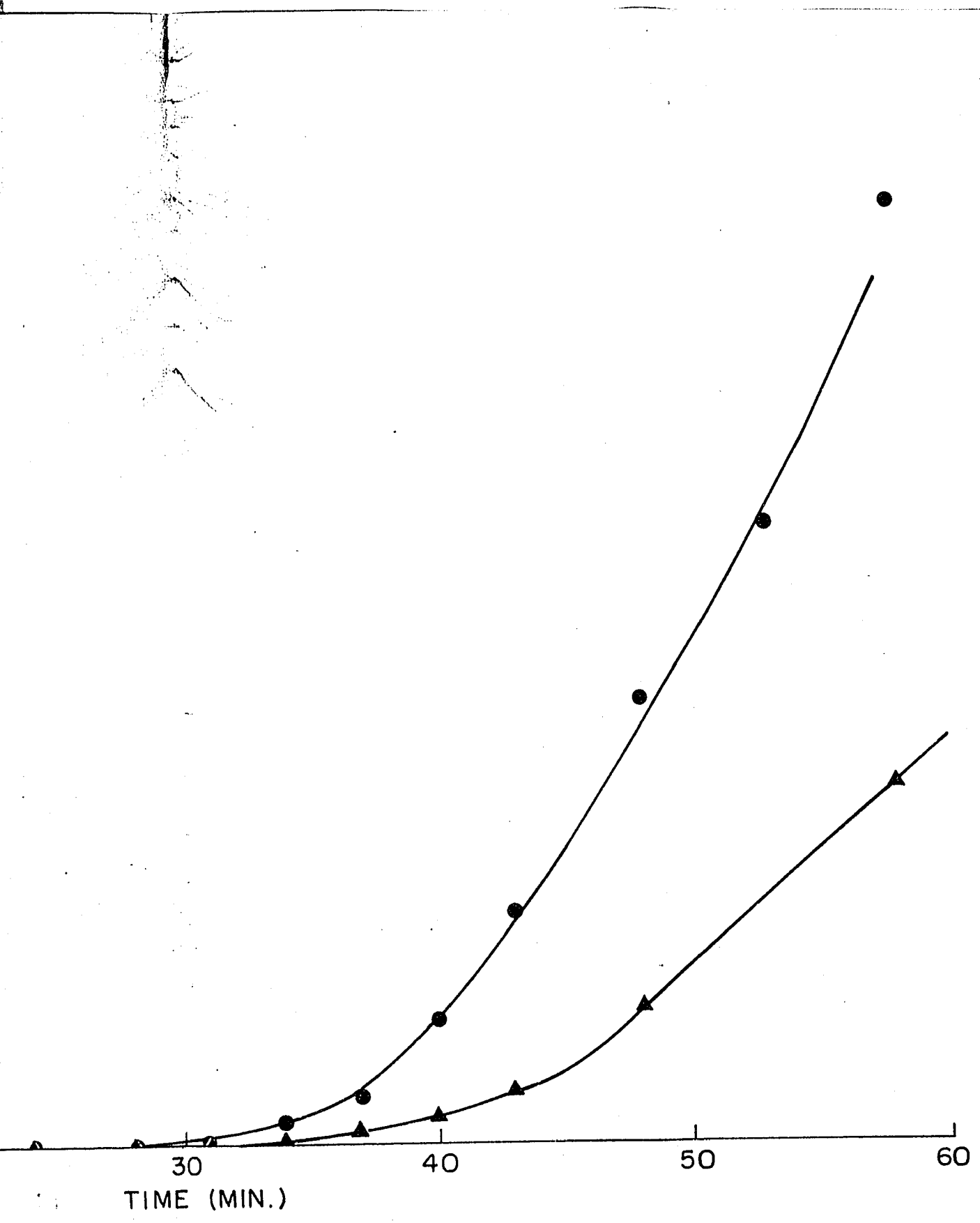
PHAGE /CELL
5MT
T
0
10
20
30
TIME (MIN.)


30
40
50
60
TIME (MIN.)

added at 7 minutes, since at 7 minutes, after infection with UV irradiated phage, the rate of DNA synthesis is zero according to previous experiments. The immediate increase in DNA synthesis upon reversal of the inhibition with tryptophane at 22 minutes is of interest. Something evidently occurred during the 15 minute period with 5-methyl tryptophane which made up approximately 5 minutes of the delay usually observed. What process is involved here is not clear.

In Figure 14, the curve for intracellular phage development for infection with UV irradiated phage again lags behind that of the control but not by the 7 or 8 minutes observed previously. This effect is probably related to the 5 minutes shortening of the DNA lag period just mentioned.

In summarizing the results of the experiments using 5-methyl tryptophane the following observations may be made. Some kind of protein synthesis takes place shortly after infection with UV irradiated as well as with unirradiated T_2r+. Protein synthesis is necessary to initiate DNA synthesis but is not necessary for its continuation. Some steps in the multiplicity reactivation process occurring after the initial protein requiring steps, but before the protein synthesis for phage coats, do not require protein synthesis. In order for active intracellular phage to appear protein synthesis is necessary, undoubtedly for the protein coats of the phage.

Further experimental work should be done with 5-methyl tryptophane in connection with UV irradiated phage in order to determine the periods in the multiplicity reactivation process requiring protein synthesis and

those not requiring protein synthesis. In this way, more light might be shed on the mechanism of phage reproduction in general.

SUMMARY OF RESULTS

A series of experiments on multiple infection of Escherichia coli B. by UV irradiated T_2r+ or T_4r+ bacteriophage was performed, with multiple infection by unirradiated phage as controls.

Multiplicity reactivation, which ordinarily takes place with the T even phages, did not take place with either T_2r+ or T_4r+ when multiple infection of bacterial cells, which were grown to a limited concentration in a synthetic glucose-salts medium with limiting glucose, was allowed to take place with phage irradiated with ultraviolet light to a survival of 0.03 to 0.003. These cells did yield new phage upon multiple infection with unirradiated phage.

Multiplicity reactivation was found to take place in cells grown in a regular glucose-salts medium, containing more than a limiting amount of glucose, if leakage and "lysis-from-without" were prevented by infecting the cells in the medium itself and not in buffer, and in cells grown in a glucose-salts medium supplemented with amino acids, vitamins, and minerals, in which case the cells could be centrifuged out and resuspended in buffer for infection without danger of leakage or "lysis-from-without."

Using one of the systems in which multiplicity reactivation does take place, multiple infection experiments were carried out with UV irradiated and unirradiated T_2r+. The total DNA, as measured by the Dische diphenylamine test on 0.3M. trichloroacetic acid precipitates of cultures

drawn at various times after infection, increased after a 3 to 5-minute lag period in the case of infection with unirradiated phage. The total DNA curve, after infection with UV irradiated T_2r+, was similar in shape but lagged behind the former DNA curve by 8 to 10 minutes in each case when the experiments were run simultaneously on separate fractions of the same bacterial culture. The UV absorbing acid soluble components from these same samples were found to increase, in general, with time after infection, and the values for infection with UV irradiated phage were the same as for infection with unirradiated phage for the first 60 minutes, after which the amount of UV absorbing acid soluble materials in the case of irradiated phage was greater. The absorption spectra of the acid soluble fractions revealed peaks at 260 mμ indicative of nucleic acid breakdown products. The differences between spectra of different samples indicated the presence of protein derivatives also.

Duplicate one-step growth experiments with multiple infection with UV irradiated and unirradiated T_2r+ gave latent periods within one or two minutes of each other and burst sizes of approximately 50 and 100 phage per cell respectively.

Premature lysis of the cells in a chloroform-cyanide mixture revealed that a similar 8 to 10-minute lag, found in the increase of total DNA in the case of infection with UV irradiated phage, was present in the appearance of active intracellular phage in this instance.

A determination of the increase in T_2r+ DNA with time, based on the presence in T even phage DNA of the pyrimidine base hydroxymethyl cytosine and the absence of cytosine, was made for simultaneous bacterial

cultures multiply infected with UV irradiated and unirradiated bacteriophage. Paper electrophoresis of the bases obtained by acid hydrolysis of DNA from samples of duplicate cultures of cells infected with UV irradiated and unirradiated T_2r+, enabled curves to be drawn for the amount of T_2r+ DNA and bacterial DNA versus time after infection in each of the two instances. The curves for bacterial DNA indicated decreases with time after infection in each of the two cases. The curves for T_2r+ DNA indicated a period of zero increase followed by a rise to a steady rate of production of DNA. The curve for multiple infection with UV irradiated phage lagged behind that for unirradiated phage by 8 to 10 minutes but had the same shape. In the same experiment, simultaneous determinations of active intracellular phage at various times after infection made possible an estimate of the pool size in terms of phage units of DNA per bacterial cell which amounted to 45 to 50 in the case of infection with unirradiated phage and in the case of UV irradiated phage.

By using 5-methyl tryptophane as an inhibitor of protein synthesis, and adding it at zero time or at a later time in simultaneous cultures of bacteria multiply infected with UV irradiated phage or with unirradiated phage, and reversing the effect at later times by the addition of tryptophane, it was determined that protein synthesis is necessary soon after infection with UV irradiated as well as with unirradiated T_2r+ in order for phage production to take place. Protein synthesis is necessary for the initiation of DNA synthesis but not for its continuation. There is evidence that some of the steps in the multiplicity reactivation process, after the initial steps, do not require protein synthesis.

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